Intramural Activities

October 1, 1993 - September 30, 1994

94 annual report

Division of

Cancer Biology, Diagnosis, and Centers

NATIONAL INSTITUTES OF HEALTH
National Cancer Institute

October 1, 1993 - September 30, 1994

Division of

Cancer Biology, Diagnosis, and Centers

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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NATIONAL CANCER INSTITUTE
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS

ANNUAL REPORT

October 1, 1993 through September 30, 1994

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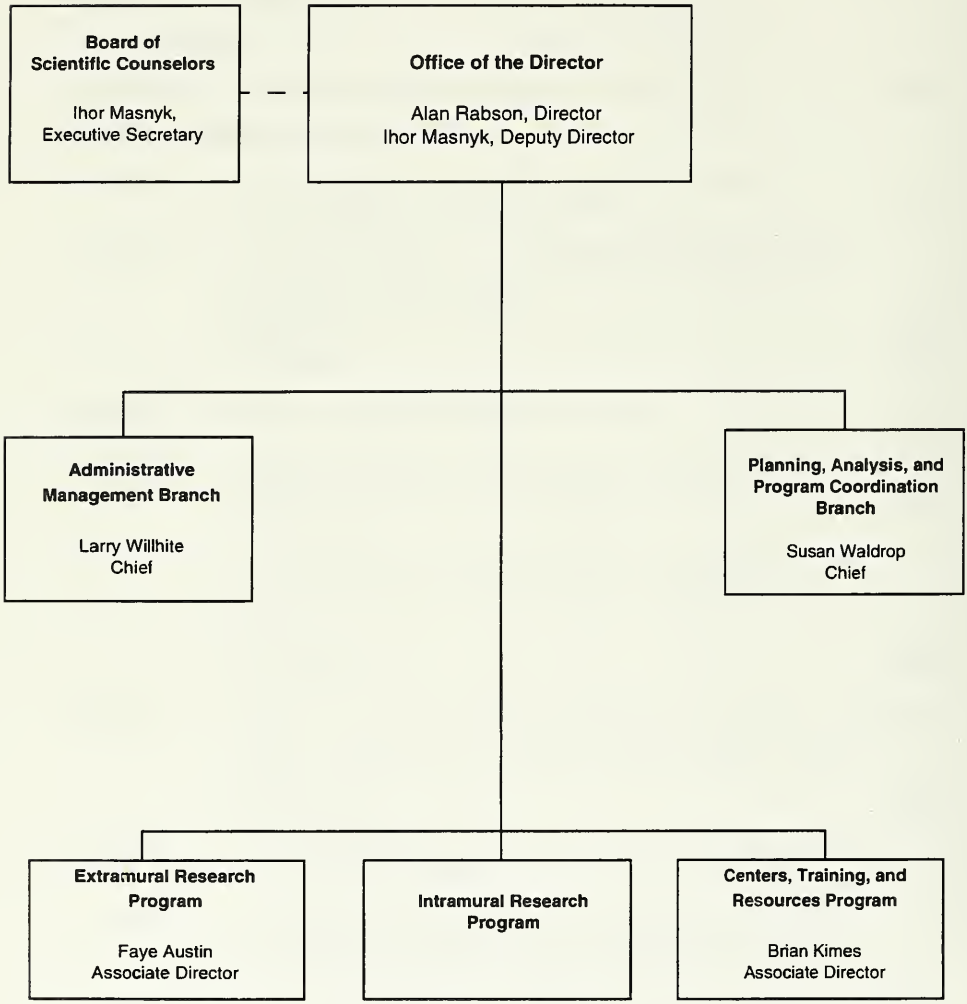

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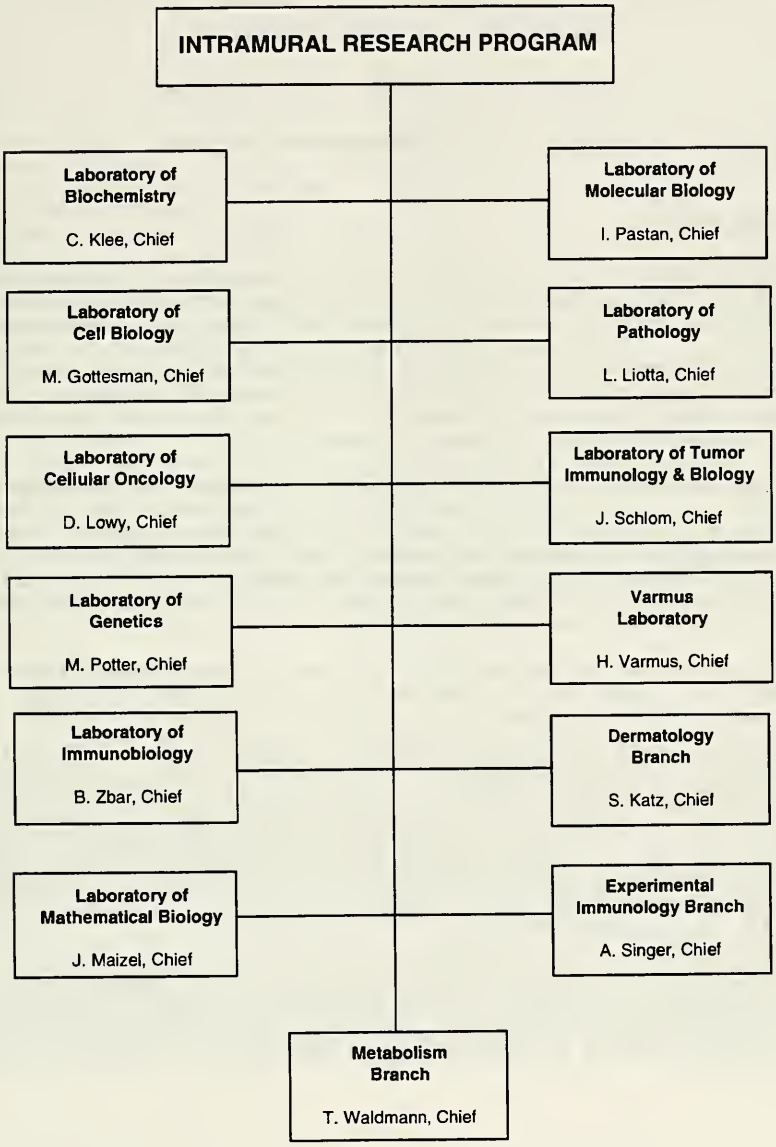

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1993 through September 30, 1994

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. 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 will focus on the research activities of the intramural research program. Information describing the activities of the other programs is included in separate volumes.

The Intramural Research Program in the Division of Cancer Biology, Diagnosis, and Centers consists of thirteen laboratories and branches at the NIH Bethesda campus and at the Frederick Cancer Research and Development Center. Each of these laboratories is directed by a scientist of international stature, four of whom are members of the National Academy of Science, two of whom are members of the Institute of Medicine, one of whom is a Nobel Laureate, and one of whom is a winner of the Lasker Award.

The DCBDC intramural laboratories are: The Laboratory of Biochemistry, directed by Dr. Claude Klee; the Laboratory of Cell Biology, directed by Dr. Michael Gottesman; the Laboratory of Cellular Oncology, directed by Dr. Douglas Lowy; the Laboratory of Genetics, directed by Dr. Michael Potter; the Laboratory of Immunobiology, directed by Dr. Berton Zbar; the Laboratory of Mathematical Biology, directed by Dr. Jacob Maizel; the Laboratory of Molecular Biology, directed by Dr. Ira Pastan; the Laboratory of Pathology, directed by Dr. Lance Liotta; the Laboratory of Tumor Immunology and Biology, directed by Dr. Jeffrey Schlom; the Varmus Laboratory, directed by Dr. Harold Varmus; the Dermatology Branch, directed by Dr. Stephen Katz; the Experimental Immunology Branch, directed by Dr. Alfred Singer; and the Metabolism Branch, directed by Dr. Thomas Waldmann.

The diversity and high quality of each of these research programs reflects the varied interests and training of the investigators within the division. Their continued interaction and exchange of ideas and technical expertise fosters a fruitful environment for the advancement of laboratory and clinical investigations.

Research conducted in the intramural laboratories covers a broad range of investigations from the regulation of gene expression in simple prokaryotic systems to clinical trials of new therapeutic agents for 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 to define the genetic changes responsible for neoplastic transformation and for the progression of cancer from localized to metastatic disease. A strong program in basic immunology explores the complex mechanisms governing the regulation of the immune response. While the aim of these studies is to further our understanding of the principles governing immune cell function, a long-term goal 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, and those elements of the immune system that can be stimulated to provide a more effective antitumor response. Already, discoveries in fundamental immunology have suggested entirely new ways to control or prevent the development of cancer. During the past year, Phase I clinical trials of recombinant vaccinia virus vaccines and a peptide vaccine for human cancer were initiated. In addition, studies of the normal immune response have led to important discoveries regarding the immune dysfunction associated with the progression from asymptomatic HIV infection to the development of AIDS.

The focus of many of the laboratories in the intramural research program of DCBDC is on areas of high priority to the NCI, such as breast cancer and vaccine development. However, a vigorous program of fundamental untargeted research is supported in the firm belief that discoveries in basic science hold the greatest promise for advances that will translate into real improvements in the treatment, diagnosis and ultimately the prevention of cancer.

Selected highlights of the research activities and major accomplishments of the Intramural Research Program are described below. 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 Biochemistry
Dr. Claude Klee, Chief

The individual research groups within the Laboratory of Biochemistry span a broad array of scientific expertise and provide a multidisciplinary approach to the study of the complex mechanisms of cellular regulation. Dr. Carl Wu and his colleagues have continued to focus their attention on heat shock gene expression, with emphasis on the structure and regulation of the heat shock transcription factor (HSF) and on the organization of the heat shock gene promoter in chromatin. Considerable advances were achieved in the past year in the biophysical studies of the HSF DNA-binding domain using the newly acquired analytical ultracentrifuge. A highlight of this group's achievements was the determination of the solution structure of the DNA-binding domain of HSF and the identification of its DNA-recognition helix in collaboration with Dr. Adriaan Bax (NIDDK). The role of cellular factors in regulating the activity of HSF was clarified, setting the stage for the identification of the upstream components of the stress signal transduction pathway. Another major

achievement was the discovery of an ATP-dependent activity necessary for the remodeling of chromatin structure. Preliminary findings from this group also indicate a novel, chromatin-related repression mechanism for the heat shock genes during the mitotic phase of the HeLa cell division cycle. The hypersensitivity to heat stress of cells blocked in mitosis may offer a therapeutic window for combining anti-mitotic drugs, such as taxol, and hyperthermia in cancer treatment.

Work in Dr. Claude Klee's group is aimed at elucidating the mechanism of stimulus response coupling mediated by Ca^{2+} and calmodulin using the calmodulin-stimulated protein phosphatase, calcineurin, as a model system. The ultimate goal of this group is to elucidate the structure of the calcineurin/calmodulin complex and to determine the molecular basis for the inhibition of calcineurin by the two immunosuppressive drugs, FK506 and cyclosporin. During the past year, Dr. Ren Hao succeeded in expressing the two subunits of calcineurin in *E. coli*. The secondary structure of the regulatory subunit, calcineurin B, has been determined in collaboration with Drs. Adriaan Bax and Jacob Anglister (NIDDK). The highlight of this year's achievements was the reconstitution of an active enzyme from the two recombinant subunits which can now be used to determine the crystal structure of calcineurin complexed with calmodulin. Site-directed mutagenesis of the recombinant enzyme will facilitate the elucidation of the mechanism of action of the new immunosuppressive drug, FK506.

Recent studies by Dr. Michael Lichten and colleagues have elucidated the roles that both chromatin and chromosome structure play in determining the sites of meiotic recombination. They showed that double-strand DNA breaks, which initiate meiotic recombination, occur exclusively at sites that display enhanced sensitivity to non-specific nuclease digestion of chromatin *in vitro*, suggesting that accessibility of DNA in chromatin controls where breaks occur. They have mapped the location of double-strand breaks at high resolution at several sites, providing further information about the substrate requirements of the break-forming activity and of the biochemical nature of the breaks themselves. They have also provided evidence that chromosome pairing precedes meiotic recombination.

Laboratory of Cell Biology
Dr. Michael Gottesman, Chief

The Laboratory of Cell Biology conducts work 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 on the regulation of encapsidation of HIV RNA.

Dr. Michael Gottesman, in collaboration with Dr. Ira Pastan in the Laboratory of Molecular Biology, has delineated one molecular basis of resistance to natural products resulting from expression of the *MDR*1 gene which encodes the 170,000 dalton P-glycoprotein (PGP), an energy-dependent multidrug efflux pump. Current studies focus on the mechanism of action of this pleiotropic transporter, and the use of vectors encoding the *MDR*1 gene for gene therapy of

cancer and inborn errors of metabolism. Analysis of this multidrug transporter has led to a model in which natural product hydrophobic drugs which interact with the transmembrane domains of PGP are removed directly from the plasma membrane. Work is underway to determine more precisely the mechanism of action of the PGP pump. Retroviral vectors encoding the *MDR*1 gene can confer multidrug-resistance on bone marrow of mice resulting in selective advantage of transduced bone marrow cells *in vivo*. This approach might be useful to protect the bone marrow of human cancer patients from the toxic effects of chemotherapy. New *MDR*1-based retroviral vectors which use an internal ribosome entry site to allow efficient expression of non-selectable genes have been constructed. This approach could allow selection of human bone marrow cells which express otherwise non-selectable genes such as those encoding glucocerebrosidase (Gaucher disease) or alpha galactosidase (Fabry disease).

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. A major effort has focused on an analysis of papillomaviruses (PV), with the aim of developing a vaccine to prevent HPV16 infection.

Drs. Douglas Lowy and John Schiller and their colleagues have used recombinant baculoviruses to produce self-assembled virus-like particles (VLP) from several human and animal PV types via baculovirus vectors. The VLPs are properly folded and induce high tier neutralizing antibodies, which are directed against conformational epitopes. Vaccines based on cottontail rabbit PV (CRPV) VLPs were able to prevent experimental CRPV infection in rabbits. Drs. Lowy and Schiller have identified HPV16 L1 clones from primary lesions that, unlike the prototype L1 used in previous studies, efficiently assemble into particles. Rabbit antibodies to the efficiently assembled HPV16 L1, but not the prototype L1, blocked the binding of HPV16 VLPs to human keratinocytes. These types of particles might be considered as candidates for a prophylactic vaccine to prevent HPV16 infection. Drs. Lowy and Schiller have analyzed the cellular effects of E2F-1, a transcription factor that is released from pRB when it binds to HPV16 E7. Their studies suggest that limited activation of E2F-1 is an important, but not the sole, function of E7.

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). The model used by Dr. Potter's group is the mouse plasmacytoma system. A major interest is in identifying genes that determine the unique susceptibility of BALB/c mice to this form of tumor induction. Two participating genes have been localized to separate regions on chromosome 4, and research is underway to place these genes in recombinational units of less than 5 centimorgans. A potentially important clue provided by the PCR detection of myc-Sα

illegitimate recombinations is that there are quantitative differences in the number of these recombinations between BALB/c and the partially resistant C.D2 MIA strain as shown by Jürgen R. Müller. This provides a basis for a 30 day test and suggests the chromosome 4 genes act much earlier than previously suspected. Dr. Beverly Mock is continuing to position chromosome 4 PCT susceptibility genes using the panel of typed backcross DNAs. She also has found genetic differences that influence PCT induction by the RIM (myc-Ha-ras) and ABL/MYC (myc-abl) retroviruses in pristane-conditioned mice.

The study of silicone gels as inducers of plasmacytoma formation has opened new questions and possibilities. Dr. Potter and his colleagues are attempting to identify the active components in the gels and determine if their primary effect is due to their immunological adjuvanticity or to direct genotoxicity. Because of the wide-spread use of these gels in implants, this work may be relevant to the study of the pathogenesis of multiple myeloma.

With Drs. Jürgen Müller and Charles Rabkin, a PCR methodology for assaying the human t(8;14) translocations involving c-myc-Su recombinations has been established, and the peripheral blood lymphocytes from 127 HIV' men are being analyzed; translocations have thus far been detected in 5 of these. The work indicates that the c-myc-Ig switch region illegitimate recombination is an early oncogenic step that is required but not sufficient to transform a B lymphocyte to full malignant potential.

Laboratory of Immunobiology
Dr. Berton Zbar, Chief

The genetic basis of human renal cell carcinoma has been the major focus of the Laboratory of Immunobiology. Their major accomplishment this year, in collaboration with Dr. Marston Linehan of the NCI Surgery Branch, has been the identification and characterization of germ line and somatic mutations in the Von Hippel Lindau (VHL) gene. This gene appears to play a major role in the pathogenesis of sporadic clear cell carcinomas of the kidney.

Drs. Burton Zbar and Michael Lerman and their colleagues have demonstrated that almost 60% of sporadic clear cell carcinomas of the kidney had somatic mutations in the VHL gene. Missense, nonsense and frameshift mutations in the VHL gene were detected in sporadic clear cell carcinomas of the kidney; large deletions were not found. About half of the VHL mutations would be predicted to truncate the predicted protein product. Mutations in the VHL gene were found in both early and advanced stages of renal carcinoma. Somatic mutations in the VHL gene were not detected in papillary renal cell carcinomas (n = 10). Dr. Zbar and his colleagues have identified mutations in 85/114 (75%) of VHL families. Missense, nonsense, frameshift and large deletion mutations in the VHL gene were detected. The mutations were distributed over a 400 nucleotide region in exons 1, 2 and 3.

Laboratory of Mathematical Biology
Dr. Jacob Maizel, Chief

Research in the Laboratory of Mathematical Biology covers a broad range of theoretical and experimental studies of biological systems which provide models for aspects of malignant and other disease processes.. These studies include molecular modeling, theoretical molecular calculations, molecular glycobiology, membrane structure and function, and physiological modeling studies. 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 using the supercomputing facilities at the Frederick Biomedical Supercomputing Center, FCRDC.

Dr. Maizel's group has focused on using their methods for RNA folding as starting points for developing methods for 3-D molecular models. These RNA models will be the basis for further studies on RNA catalytic and binding activities and on RNA-protein interactions. An initial, detailed 3-D model of domain II of the rev response element (RRE) of HIV is completed. This model indicates bending of the RNA-helix, conformational distortion of the sugar-phosphate backbone at two predicted non-Watson-Crick base pairs (G:G and G:A), and widening of the major groove, all of which may be necessary for interaction with rev protein.

Dr. Robert Jernigan's group is studying conformational variation of DNA and DNA-protein bending. Ways to treat RNA folding in three dimensions have also been considered. Transfer RNA was used as a test molecule to investigate large numbers of possible arrangements. In the largest generation of over 2 million conformations, 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 and quadruple strand helices and their interactions with monovalent cations are being investigated.

Using a variety of biophysical, biochemical and molecular biological techniques, Dr. Blumenthal has continued to analyze steps in viral envelope protein-mediated fusion including the kinetics of HIV entry into cells. The parameters and "design principles" derived from these studies 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. Since transmission of HIV-1 between cells is thought to be associated with cell membrane fusion, an understanding of the mechanism of viral fusion might lead to the development of new anti-viral drugs for AIDS. Dr. Dimiter Dimitrov has initiated a project to quantify stages of the HIV-1 life cycle, using a variety of biophysical, cellular and molecular biology techniques combined with mathematical modeling to understand how replication kinetics is affected by viral and cellular structures. A novel approach for quantitation of HIV-1 infection kinetics has been developed which allows the determination of a critical parameter of a spreading virus infection, the number of infecting virions produced by an infected cell and transmitted to uninfected cells, as well as the time required for one cycle of infection.

Laboratory of Molecular Biology
Dr. Ira Pastan, Chief

The Laboratory of Molecular Biology uses genetics, molecular biology, and cell biology to study gene activity and cell behavior. One major goal is to develop new approaches to the treatment and diagnosis of cancer, AIDS and other human diseases. Another is to understand how cell division and gene activity is regulated.

Dr. Ira Pastan and his colleagues have designed and produced both conventional immunotoxins and fully recombinant toxins for the treatment of human cancer and AIDS. Immunotoxin LMB-1 (a conventional immunotoxin composed of MAb B3 and a mutant form of *Pseudomonas* exotoxin, PE) is in clinical trials on patients with colon, breast, stomach and esophogeal cancers. LMB-7 is a smaller recombinant immunotoxin also made with the B3 antibody. LMB-7 entered trials in the same patient population in 1994. Other immunotoxins directed at the erbB2 oncoprotein and at the IL2 receptor present on lymphomas and leukemias will enter the clinic in 1995. TP40 (TGFα fused to a 40,000 MW fragment of PE) is being evaluated in patients with bladder cancer. A very active recombinant toxin directed at the IL4 receptor present in kidney cancers has recently been made. Recombinant immunotoxins with improved properties have been made which are (1) smaller and therefore able to penetrate into tumors better, (2) less immunogenic, and (3) more stable and easier to produce. Increased stability was achieved by using a combination of molecular modeling and genetic engineering to insert a disulfide bond at a conserved location in the framework region.

Drs. Susan Garges and Sankar Adhya are continuing their studies of the mechanisms of positive control of gene transcription by cAMP receptor protein (CRP), in the *lac* promoter of *Escherichia coli*. A number of experiments from their laboratory and other laboratories have provided evidence suggesting that activation of transcription initiation requires a protein-protein contact between DNA-bound cAMP.CRP complex and RNA polymerase. Drs. Garges and Adhya have now obtained evidence that shows that transcription activation mediated by DNA-bound cAMP.CRP also requires a structural change in the DNA. They have proposed that a cAMP.CRP induces a structural change in DNA that is transmitted to the promoter. Distortion in the double helical polynucleotide structure either by creating a two to ten base gap in one of the two strands or by the use of mismatched base-pairs in the segment of DNA between the cAMP.CRP binding site and the promoter inhibits transcription activation.

Molecular chaperones participate in protein folding pathways by recognizing and modulating partially folded proteins during protein synthesis, oligomeric structure formation, complex and aggregate disassembly and protein degradation. Drs. Susan Wickner and Dorota Skowyra have continued their studies of the mechanism of action of molecular chaperones and their role in DNA replication. They found that three *E. coli* heat shock proteins, DnaJ, DnaK, and GrpE are required for plasmid P1 DNA replication *in vitro*, and that DnaJ and DnaK activate the sequence specific DNA binding of the P1 initiator protein, RepA, by converting RepA dimers to monomers. They also discovered that ClpA, the regulatory component of the ATP-dependent ClpAP protease,

activates the sequence specific DNA binding activity of RepA. These and other results demonstrate that ClpA is a new molecular chaperone.

Laboratory of Pathology
Dr. Lance Liotta, Chief

The Laboratory of Pathology integrates anatomic pathology service and training with programs in molecular pathology research. The Laboratory is responsible for diagnostic services in anatomic pathology, surgical pathology, exfoliative cytology, fine needle aspiration, immunohistochemistry, hematopathology, and electron microscopy for the NIH Clinical Center. A fully accredited 4-year residency program is provided for 9 residents and 3 fellows.

The goal of Dr. Kathleen Kelly's laboratory is to identify proteins that regulate the activation process in lymphoid cells and to characterize the biochemical basis of their activity. One such induced protein is PAC1, a hematopoietic cell-specific, nuclear protein phosphatase with dual specificity for threonine and tyrosine. Dr. Kelly's lab has used several approaches to demonstrate that PAC1 is a physiologically-relevant MAP kinase phosphatase. MAP kinase activation is a central component of signal transduction pathways initiated by several growth and differentiation factors and oncogenes. The initial activation of MAP kinase is reversed by PAC1 assuring the transient nature of this early signal transduction pathway.

A major research focus within the Laboratory of Pathology is on studies of tumor cell invasion and metastasis, with special emphasis on breast cancer. The goal of these studies is to further elucidate the mechanisms responsible for the development of the metastatic phenotype and to suggest new strategies for the improved iagnosis, treatment and prevention of cancer. Dr. William Stetler-Stevenson's group has continued its study of TIMP-2, a member of the tissue inhibitor of the metalloproteinase family that can block the degradative activity of procollagenase A produced by invasive tumor cells. New studies suggest an anti-angiogenic role for TIMP-2. Dr. Stetler-Stevenson's group has demonstrated that TIMP-2, but not TIMP-1, selectively blocks the mitogenic response of endothelial cells to bFGF. The use of the synthetic metalloproteinase inhibitor, BB94, confirmed that this effect was unique to TIMP-2 and was not mediated by metalloproteinase inhibitory activity of this protein. Preliminary results suggest that these effects are mediated by a specific and saturable TIMP-2 receptor. Current efforts are aimed at isolation and characterization of this receptor. Further studies suggest that alterations in the matrix metalloproteinase and TIMP balance may disrupt not only ECM proteolysis but cell adhesion and migration required for effective cell invasion.

Dr. Mary Stracke is cloning the gene for a potent new motility stimulating cytokine, autotaxin, which stimulates a pertussis toxin-sensitive motility response in tumor cells. Anti-peptide antibodies against selected autotaxin peptides have been produced and are being utilized for biochemical and histochemical studies of autotaxin. Dr. Marie Beckner has cloned the gene for AAMP-1, a newly identified transmembrane protein that regulates cell locomotion.

CAI (carboxyamido-triazole) has served as a unique tool to study novel cell signaling pathways and their biologic consequences. Dr. Elise Kohn's group has demonstrated that CAI inhibition of calcium-mediated signaling events is concordant with inhibition of malignant proliferation in vitro and that the complete molecule is necessary for activity. Recent work demonstrates that CAI also inhibits angiogenesis. It may also drive the inhibition of myelopoiesis and erythropoiesis as demonstrated in studies of the bone marrow effects of CAI. The Phase I clinical trial (MB281) of orally administered CAI for patients with refractory solid tumors began accrual in March 1992 and is nearing completion; 38 patients have received CAI. Preclinical efficacy and toxicity studies for chemoprevention use of CAI have begun and are promising. In vitro supra-additive efficacy of the combination of CAI preceeding paclitaxel has been seen; a Phase I clinical trial will be initiated to test this combination.

The Women's Cancers Section (WCS), under the direction of Dr. Patricia Steeg, performs basic molecular biology and biochemical investigations of women's cancers, primarily breast cancer. Major projects focus on elucidating the molecular basis of breast preneoplasia, and on delineating the negative regulatory role of the extracellular matrix component thrombospondin (TSP) in tumor progression. A third project concerns the tumor metastasis suppressor gene, *nm23*, previously discovered by Dr. Steeg. Recent studies suggest that a serine phosphorylation pathway may be involved in Nm23's regulatory effects. The genomic organization and regulation of Nm23 gene expression are also under investigation. Data to date indicate that transcriptional regulation, and not allelic deletion of Nm23 is likely responsible for the reduced expression observed in aggressive breast carcinomas. In collaboration with the Developmental Therapeutics Program, DCT, NCI, Dr. Steeg's group has identified 45 pharmacological agents from a bank of 32,000 which are preferentially cytostatic/cytotoxic *in vitro* on low Nm23 expressing human breast and melanoma tumor cell lines. Planned investigations will determine the *in vitro* effects of each agent on Nm23 expression and biochemistry, and on standard metastasis related assays. These data will be used to prioritize agents for *in vivo* testing, and eventual consideration for clinical trials.

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. Major projects involve the development of monoclonal antibodies (MAbs) directed against human carcinoma associated antigens, and the construction and evaluation of recombinant vaccines for the active specific immunotherapy of human carcinomas.

Drs. Jeffrey Schlom and Judy Kantor and their colleagues have led a major effort aimed at the construction of recombinant vaccinia viruses containing human tumor associated genes and the development of recombinant forms of these gene products in baculovirus. Their initial studies with a recombinant vaccinia virus containing the human CEA gene have demonstrated that this construct is immunogenic and safe in both rodents and primates, and that it

will elicit good anti-tumor responses in a rodent model. A recombinant CEA vaccine has been evaluated in 26 patients with CEA expressing tumors in a Phase I clinical trial at the NCI. The results showed that the recombinant CEA vaccinia virus can be safely administered to cancer patients with no toxicity and that this vaccine can serve as an immunogen for repeated exposures to a specific tumor associated antigen. Clinical and immunological evaluations for the presence of CEA specific humoral and cell mediated responses are being performed. A recombinant PSA vaccinia virus has also been constructed and evaluated for its safety and immunogenicity in a murine tumor model expressing human PSA and in male rhesus monkeys. Studies are underway to evaluate these animals for PSA specific cell mediated responses. Dr. Kwong Tsang's group has investigated the primary response to CEA peptides of peripheral blood leukocytes (PBL) derived from normal individuals and cancer patients. T-cell lines to CEA peptides were established from patients with carcinoma using 9-mer or 10-mer CEA peptides. These T-cell lines were peptide specific as assayed by cytokine release and cytotoxicity assays. Further characterization of these cell lines is in progress.

Dr. Robert Callahan's group has continued an on-going program aimed at determining those genetic alterations in primary breast tumor DNA that have a statistically significant association with the patient's history, characteristics of the tumor, and the patient's prognosis. The most frequent type of mutation is loss of heterozygosity (LOH) at specfic regions of the cellular genome in tumor DNA. Their data suggest that on chromosome 1p there are at least two tumor suppressor genes located at 1p13-p21 and 1p32-pter that are affected by LOH in primary breast tumors. Similarly, five regions of chromosome 17 appear to be independently affected by LOH. BRCA1, the gene associated with familial breast cancer, is located on chromosome 17q21. In sporadic breast tumors Dr. Callahan has defined a 150kb within this region which is frequently affected by LOH. Currently he is attempting to identify the target gene within this region and determine its relationship to BRAC1.

Varmus Laboratory
Dr. Harold Varmus, Chief

The Varmus Laboratory uses molecular and genetic approaches to understand the normal and pathogenic roles of genes implicated in human and animal cancers. It is convenient to consider their activities in three categories:

(1) The src gene family. The Varmus Laboratory is currently emphasizing the use of mice with targeted mutations in the four src-like genes expressed in the monomyelocytic lineage--src, fgr, hck, and lyn--to explore the normal functions of these genes. At least three issues are under study: What is responsible for the loss of natural immunity in mice lacking both hck and fgr? Can we protect src-deficient mice from osteopetrosis by expressing a src transgene in the macrophage lineage? What accounts for the defect in hematopoiesis observed in mice deprived of both hck and src? In pursuing these questions, they are also making use of recent findings that suggest that some normal function of src protein can be attributed to the aminoterminal half, rather than to the kinase domain.

(2) The Wnt gene family. A variety of approaches are being utilized to seek receptors for the secretory glycoproteins encoded by Wnt genes--genes that have been implicated in a broad collection of developmental events, as well as tumor formation. These studies include efforts to bind Wnt fusion proteins to the surface of cells expressing receptors and structural and functional screens of cDNA libraries, using assays in mammalian cells and frog embryos. In conjunction with these efforts, they are examining the potential signalling pathways activated by Wnt proteins and some candidate receptor genes that have been recently isolated.

(3) Mammary carcinogenesis. Transgenic mice that express the Wnt-1 gene in the mammary gland and develop mammary hyperplasia and carcinoma are being used to study the multi-step process required for the full oncogenic phenotype. An activated Wnt gene can collaborate with activated FGF genes or with inactivated p53 genes to accelerate tumorigenesis; other collaborating genes are being sought by genetic crosses and other methods, including insertional mutagenesis with mouse mammary tumor virus. They are also using analysis of karyotypes, specific genes, and the physiological behavior of mammary cells to understand the contributions made by each event in tumorigenesis. Furthermore, they are examining the role of tumor immunity in the generation of these neoplasms.

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. Individual laboratory groups are studying the immunopathologic mechanisms involved in inflammatory and neoplastic skin diseases, the role of cell-surface adhesion molecules in Langerhans cell-T cell activation, mechanisms of keratinocyte adhesion to epidermal basement membrane, the molecular basis of autoimmune disease, new treatment approaches for skin cancer and disorders of keratinization, 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 major area of study in Dr. Stephen Katz's laboratory is the role of the epidermis as an immunological organ, with special emphasis on the role of the Langerhans cell. To better understand the nature of the precise epitopes generated after hapten-Langerhans cell interaction, they have begun assessing the ability of TNP-modified I-A binding peptides to activate hapten-specific CD4' T cells. Their results indicate that hapten-modified MHC class II binding peptides generate epitopes recognized by hapten-specific CD4' T cells and that precise positioning of hapten molecules is required for optimal CD4' T cell recognition. These findings provide insight into how haptens are recognized by T cells in contact sensitivity and should facilitate the study and design of specific therapies for the manipulation of hapten-specific CD4' T cell responses.

During the past year, Dr. Mark Udey has shown that the expression of E-cadherin appears to be a general feature of Langerhans cells. Work is underway to develop a model system that will permit studies of mechanisms that regulate the level of expression and affinity of E-cadherin molecules on the surfaces of Langerhans cells or related cells.

Dr. John Stanley's group has continued its studies of autoantibody-mediated skin diseases. They have found that autoantibodies from these patients, who develop blistering diseases due to defects in epidermal cell adhesion, are directed against adhesion molecules. They have characterized the antigens defined by these diseases, and have initiated studies of their cell biologic function. Recent studies have shown that BP antigen 1 (BPAG1) is in the plaque of the hemidesmosome. Current studies are aimed at determining the function of various subdomains of BPAG1 by transfecting CDNA that encode them into eukaryotic cells. The deduced amino acid sequence of the pemphigus vulgaris (PV) antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules and is closely related to the pemphigus foliaceus (PF) antigen. Current studies are aimed at dissecting the functions of various extracellular and intracellular domains of PV antigen in cell adhesion and in binding to molecules (e.g., plakoglobin) in the desmosomal plaque.

Experimental Immunology Branch
Dr. Alfred Singer, Chief

The Experimental Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis on lymphocyte differentiation and regulation, cell biology of immune responses, signal transduction, structure, regulation and function of genes involved in immune responses, lymphocyte effector function, developmental biology, transplantation biology, and tumor immunology. The Branch's flow cytometry laboratory supports 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.

Dr. Alfred Singer's laboratory continues to examine the intra-thymic differentiation of functionally and phenotypically distinct T cell subsets as well as their interaction with thymic epithelium. Their studies suggest that T cell receptor positive (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. Dr. Singer and his colleagues have demonstrated both TCR-mediated and non-TCR-mediated requirements for the transition of precursor thymocytes of the $CD4^-CD8^{lo}$ phenotype into $CD4^+CD8^+$ double positive thymocytes. They have identified a novel regulatory mechanism that acts on developing precursor thymocytes independently of TCR and is mediated by cortical thymic epithelial cells (TEC). The contribution of non-TCR mediated signals to thymocyte negative selection has been delineated by analysis of requirements for the in vitro induction of apoptosis. It was found that TCR engagement alone does not efficiently induce apoptosis of double positive thymocytes. However, signals generated by simultaneous engagement of TCR and the costimulatory molecule

CD28 deliver a potent apoptotic signal. These results provide a molecular basis for differences among cell types in their ability to mediate negative selection of developing thymocytes.

Dr. Gene Shearer's laboratory is investigating human T helper cell (TH) function in: a) asymptomatic HIV-infected (HIV+) individuals, b) HIV-exposed individuals who exhibit no evidence of infection, c) patients with systemic lupus erythematosus (SLE), and d) cancer patients. It was found that HIV+ individuals, SLE patients, and patients with untreated Hodgkins disease and prostate cancer 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. 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.

The identification of costimulatory molecules involved in T cell activation has been carried out in the laboratory of Dr. Richard Hodes. A monoclonal antibody (GL1) was generated which identifies a molecule expressed on activated B cells, macrophages, dendritic cells, and activated T cells. It identifies a ligand for the T cell activation molecule CTLA4 that is distinct from the previously described B7 (now B7-1) and is designated as B7-2. B7-2 is encoded by a gene related to but distinct from that encoding B7-1. Anti-B7-2 MAb GL1 inhibited accessory cell-dependent responses of T cells in vitro and in vivo, indicating that B7-2 is functionally a costimulatory molecule for T cell-dependent immune responses.

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. Dr. Henkart's laboratory has also been studying the mechanism of programmed cell death in lymphocytes. Using protease inhibitors, particularly those directed towards calpain, they have identified a cell death pathway involved in the antigen-induced death of mature T lymphocytes. Calpain inhibitors partially restore defective T helper proliferative responses of cells from HIV' donors, as well as other activation responses limited by TcR-induced death.

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. Dr. Waldmann's studies have focused on the role played by the IL-2/IL-2R system in normal and abnormal T-cell function and the use of these insights to develop IL-2R directed therapy for leukemia/lymphoma. The IL-2R involves three IL-2 binding subunits, 55 kDa (IL-2Rα), 70-75 kDa (IL-2Rβ) and 64 kDa (IL-2Rγ). During the past year Dr. Waldmann has defined a 15 kDa lymphokine (IL-T) that stimulates T-cell

proliferation and the activation of large granular lymphocytes; further studies revealed that IL-T requires IL-2Rβ and IL-2Rγ expression for its action. Dr. Waldmann and his colleagues completed a clinical trial with ^{90}Y-anti-Tac for patients with HTLV-I-associated ATL. Twelve of the 18 patients in this trial manifested a partial or complete remission following ^{90}Y-anti-Tac therapy. Dr. Waldmann has extended these studies to ^{90}Y linked humanized (rather than murine) anti-Tac for treatment of an extended array of human leukemias and lymphomas. Furthermore, using a tumor model in nude mice, Dr. Waldmann demonstrated the efficacy of the α-particle-emitting radiolabeled murine anti-Her-2/neu monoclonal antibody (^{212}Pb-AE1) in the prevention of development of human ovarian SK-OV-3 tumors that express Her-2/neu receptors.

Dr. Jay Berzofsky's research has focused on elucidating 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 found in murine studies that peptide vaccines for HIV can be made more potent or more broadly effective by selective introduction of mutations that improve binding to MHC or T-cell receptors. He has developed synthetic vaccines for HIV that have broadly reactive HIV helper epitopes combined with CTL and neutralizing antibody epitopes, and has shown the importance of covalent linkage of helper and CTL epitopes on the same molecule for induction of CTL. These vaccine constructs have been prepared for human phase I immunotherapy trials. Dr. Berzofsky demonstrated the profound effect of cytokine imbalance on CTL activity and viral clearance in an animal model, and the ability to overcome the downregulation of human helper T cell responses in the blood of HIV infected individuals in vitro by use of another cytokine, IL-12. He has developed peptide cancer vaccines inducing CTL immunity to mutant p53 expressed in cancer cells, and showed in animal models some degree of protection against a tumor. In this system, he has shown the usefulness of interferon-gamma in sensitizing tumor cells for lysis by CTL. The first human patient has been treated in a phase I/II clinical trial of this mutant p53 or ras peptide vaccine approach to treating cancer.

SUMMARY STATEMENT

LABORATORY OF BIOCHEMISTRY

DCBDC, NCI

OCTOBER 1, 1993, TO SEPTEMBER 30, 1994

INTRODUCTION

The fifteen groups which constitute the Laboratory of Biochemistry lead different but complementary research programs. They provide a broad array of expertise in molecular biology, genetics, microbiology, protein biochemistry and immunology with the common goal of solving basic mechanisms of cellular regulation. It has become evident during the past few years that a multidisciplinary approach is essential for the dissection of the complex networks leading from the cell surface to the nucleus. These range from protein structure to development which are conserved from bacteria to flies and vertebrate animals.

Every effort is made in the Laboratory of Biochemistry to maintain and extend the diversity and high quality of science required for this multidisciplinary approach. At the same time, some of us have taken advantage of the proximity of a first class Structural Biology Laboratory at NIH to pursue fruitful collaborative projects with Ad Bax and his colleagues (NIDDK). Common approaches to the different research projects have fostered stimulating interactions with members of the laboratory as well as with many NIH scientists. The excellent attendance at our daily seminars and the recruitment of several well-trained and dedicated post-doctoral fellows speaks well for the stimulating environment of the laboratory.

Particularly noteworthy this year were the original contributions made by Carl Wu and his colleagues in the dissection of the heat stress signal transduction pathway and the novel findings of Michael Lichten concerning the mechanism of meiotic recombination in yeast.

These accomplishments would not have been possible without the generous support and encouragement of our Scientific Director, who again this year allowed us to update our equipment with the purchase of a confocal microscope.

THE CONTROL OF GENE EXPRESSION

Different aspects of the control of gene expression are being investigated by seven independent groups. All have been successful in keeping up with this fast developing area of research.

The regulation of heat shock gene expression, with emphasis on the structure and regulation of the heat shock transcription factor (HSF) and on the organization of the heat shock gene promoter in chromatin, continues to be the focus of attention of Carl Wu and his colleagues. Considerable advances were achieved in the past year in the biophysical studies of the HSF DNA-binding domain using our newly acquired analytical ultracentrifuge. A highlight of this group's achievements was the determination of the solution structure of the DNA-binding domain of HSF and the identification of its DNA- recognition helix in collaboration with Adriaan Bax (NIDDK). The role of cellular factors in regulating the activity of HSF was clarified, setting the stage for the identification of the upstream components of the stress signal transduction pathway. Another major achievement was the discovery of an ATP-dependent activity necessary for the remodeling of chromatin structure. This finding, published as a main article in Nature, was quoted as a key paper in the field by the Journal of NIH Research. Preliminary findings

from this group also indicate a novel, chromatin-related repression mechanism for the heat shock genes during the mitotic phase of the HeLa cell division cycle. The hypersensitivity to heat stress of cells blocked in mitosis may offer a therapeutic window for combining anti-mitotic drugs, such as taxol, and thermotherapy in cancer treatment.

During the past year, Mark Mortin has expanded his genetic and molecular analysis of strong and mild suppressor mutations in the second-largest subunit of RNA polymerase II and their interactions with two regions in the largest subunit. The immediate goal of this work is to test the hypothesis generated from his molecular model of structural conservation between the DNA binding cleft of RNA polymerase II, as identified by this mutation, and DNA polymerase I. Two new mutations in the largest subunit that interact with only the strong suppressor mutations in the second-largest subunit were identified. These are predicted to also reside within the putative DNA binding cleft. A gene encoding a mutant of the second-largest subunit harboring both mild and strong suppressor mutations, transformed back into *Drosophila* , acted as a strong suppressor, and not like a mild suppressor, but did not rescue null mutations in the second largest subunit. This demonstrates the essential function of the suppressor domain and further documents the advantages of genetic analysis to analyze the interactions between the two largest subunits of RNA polymerase II.

Bruce Paterson and his colleagues continued to study 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 on 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 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.

Another member of Bruce Paterson's group, Deborah Hursh, carried out an independent study of the *Drosophila decapentaplegic (dpp)* gene which encodes a TGF-β family member that participates in a signal transduction pathway leading to embryonic midgut morphogenesis. She had previously shown that this pathway requires the action of the homeotic transcription factor *Ubx* and the product of the *dpp* gene itself in an indirect autoregulatory feedback loop. The dissection of the *Ubx* -responsive promoter region of *dpp* led to the identification of an 812 bp region in the 5' end of the gene that directs spatially correct expression of a reporter construct. A "double switch" experiment was performed whereby *Ubx* binding sites are mutated to respond to a *bicoid*-type homeodomain recognition helix, and a *Ubx* c-DNA is modified to contain the corresponding recognition helix (glutamine 50 to lysine 50). When these constructs were introduced into the germ line of flies this altered *Ubx* c-DNA activated these mutated sites *in vivo* demonstrating that the action of *Ubx* on *dpp* represents a direct interaction of *Ubx* protein on *dpp* cis-regulatory DNA. It was found, however, that alteration of a particular homeodomain recognition site within the 812 bp reporter extinguished expression in a non-*Ubx* dependent manner. This particular binding site, while conforming poorly to the consensus sequence of the Ubx recognition site, is a good site for the homeodomain transcription factor *pbx-1*, whose homolog in flies is the product of the *extradentical* (*exd*) gene. It is therefore possible that *exd* is a necessary co-factor for *Ubx* activation of *dpp*.

The research program of Charles Vinson is centered on understanding protein-protein interactions in an attempt to design novel interactions that can be used in both genetic and pharmaceutical contexts. His

present work focuses on the bZIP family of transcription factors. These proteins dimerize through a leucine zipper motif and the dimers are stabilized by sequence specific interactions with DNA. An important thrust of his work is to understand in biophysical detail the structural rules that regulate the stability and dimerization specificity of the leucine zipper dimerization domain. A systematic mutagenic study has helped identify amino acids of the leucine zipper dimerization region that contribute to both dimer stability and specificity. The results have been used to formulate rules to design dominant-negative C/EBP proteins that function in both biochemical and transient transfection assays. Presently, transgenic mouse models are being developed in which the dominant-negative proteins are targeted to different organs to examine the biological function of the C/EBP family members.

Edward Kuff's group previously identified a 150 kDa heterodimeric protein, designated EBP-80, which stimulated the promoter activity of Intracisternal A particles (IAP) LTRs *in vitro*. EBP-80 subsequently proved to be identical to Ku, a DNA-binding protein found in nuclei of mammalian cells. EBP-80 binds to ends of duplex DNA and to nicks and larger single strand gaps, stem-loop configurations, and internal regions of strand separation (bubbles). EBP-80 helps to regulate transcription of U1 and rRNA *in vitro*. It was also identified as the DNA-binding and activating subunit of a DNA-dependent protein kinase, DNA-PK, which can phosphorylate a number of transcriptional regulatory proteins including p53, c-*jun*, *cfos*, and SV40 T-antigen. Dr. Morozov, in collaboration with Dr. C. Anderson of Brookhaven Laboratory, has shown that stimulation of DNA-PK by various DNA constructs closely paralleled their affinity for EBP-80. Preliminary evidence for the cotranslocation of EBP-80 and DNA-PK to the nucleus in response to cell-cell contact further supports the suggestion that EBP-80 acts as a subunit of DNA-PK. Recently, other investigators reported that Ku bound to the RNA transcript of the HIV *tar* sequence. The *tar* transcript is thought to assume a stem-loop configuration which is the binding target of the viral *tat* gene product. Using plasmid constructs containing *tar* and other palindromic sequences in sense and antisense orientation, as well as plasmids encoding random sequences of equivalent length, Dr. Morozov is asking whether DNA-PK is activated by binding to the *tar* RNA and, if so, to what level and with what degree of sequence or configurational specificity. Kira Lueders' efforts are centered on the elucidation of the molecular basis for the selective activation of various members of the IAP gene family. She is also using subclass-specific IAP oligonucleotide probes for multilocus genomic mapping on mouse chromosomes.

The long term goal of Beverly Peterkofsky and her colleagues is to understand the intricate mechanism of the regulation of the synthesis of extracellular matrix components, collagen and proteoglycans. During the past year this group has shown that during fasting, insulin-like growth factor binding proteins (IGFBPs) 1 and 2 may inhibit collagen gene expression in connective tissues mainly by endocrine mechanisms, except for bone, where IGFBP-2 could also act by an autocrine or paracrine mechanism.

REGULATION OF CELLULAR PROCESSES

Molecular mechanisms of Ca^{2+}-mediated signal transduction, secretion, cell division, and immune responses are the focus of attention of four other groups.

Work in Claude Klee's group is aimed at elucidating the mechanism of stimulus response coupling mediated by Ca^{2+} and calmodulin using the calmodulin-stimulated protein phosphatase, calcineurin, as a model system. The ultimate goal of this group is to elucidate the structure of the calcineurin/calmodulin complex and to determine the molecular basis for the inhibition of calcineurin by the two immuno-suppressive drugs, FK506 and cyclosporin. During the past year, Ren Hao succeeded in expressing the two subunits of calcineurin in *E. coli*. The secondary structure of the regulatory subunit, calcineurin B, has been determined in collaboration with Drs. Adriaan Bax and Jacob Anglister (NIDDK). The highlight of this year's achievements was the reconstitution of an active enzyme from the two recombinant subunits which can now be used to determine the crystal structure of calcineurin

complexed with calmodulin while site-directed mutagenesis of the recombinant enzyme will facilitate the elucidation of the mechanism of action of the new immunosuppressive drug, FK506.

Paul Wagner and his colleagues studied the stimulation of catecholamine secretion in rat pheochromocytoma PC12 cells by Ca^{2+} and GTP-binding proteins. Their studies of the role of 14-3-3 proteins in Ca^{2+}-dependent secretion led them to demonstrate that, contrary to what has been reported by other investigators, 14-3-3 proteins do not regulate protein kinase C activity, and do not possess phospholipase A_2 activity. Thus, the precise role of these proteins in secretion remains to be determined. This group also showed that stimulation of secretion by GTP analogs results from their binding to and activation of a low molecular weight GTP-binding protein which appears to directly regulate exocytosis. Preliminary evidence that this GTP-binding protein interacts with nucleoside diphosphate kinase may help to elucidate the important role previously proposed for this enzyme in metastasis.

Major efforts are being made by Shelby Berger's group to elucidate the role of prothymosin alpha in cell division. This small, acidic, highly abundant, nuclear protein is absolutely required for cell division. In order to study both the regulation of the gene and the phosphorylation pattern of the protein, a new method of constructing mutants and manipulating DNA was devised. The technique makes use of a class of restriction endonucleases called hapaxomers which produce DNA with unique termini. Ligation of such DNA results in virtually error-free reassembly of the original structure or in the efficient insertion of mutant fragments as desired. Using this technique, the group has found that the prothymosin alpha gene has a potent promoter which can be down regulated by sequences in the first intron. They also found that the myc proto-oncogene protein does not regulate transcription of the gene, contrary to the findings of others. Mutants of the prothymosin alpha gene which code for proteins deficient in serine and threonine residues will be used to characterize the unusual phosphorylation mechanism of prothymosin alpha.

Michael Mage's group, working on recombinant single chain class I MHC molecules, has extended and generalized their findings of expression and biological activity of these molecules, by preparing single chain $H\text{-}2L^d$ and HLA-A2.1 molecules in addition to their original $H\text{-}2D^d$ molecule. In collaboration with David Margulies and Shozo Sakuma (LI, NIAID), they have developed a bacterial expression system for single chain MHC molecules, and have prepared an MHC-Ab conjugate that correctly orients the MHC molecule for subsequent incorporation into an immunogenic heteroconjugate.

THE ORGANIZATION OF THE HUMAN GENOME

Several new genes have been mapped by Wesley McBride and his colleagues including some which are candidate genes for genetic diseases. A recently cloned somatostatin receptor (SSTR4) has been localized to 16p13.3. Linkage analysis indicates that it is in very close proximity to the locus for polycystic kidney disease (PKD1) and studies are in progress to determine whether this gene is involved in PKD. Several other genes involved in cartilage and bone development or differentially expressed in skin have also been localized and efforts are under way to evaluate their possible role in diseases. Better methods to isolate STRPs at gene loci have been developed and are in use for disease gene mapping, particularly in small families.

Dean Hamer's group studied the genetics of complex human characteristics and diseases. They have assembled an impressive set of reagents to investigate the role of inheritance in a variety of human attributes including sexual orientation, psychological traits, alcoholism, HIV susceptibility and Kaposi's sarcoma.

In Maxine Singer's laboratory, work has continued in the *cis*-regulatory elements controlling cell-type specific transcription of L1Hs in human teratocarcinoma cells. In the past year, attention was focused

on a region between residues 60 and 100 of the 900 bp long UTR which contains sequences important for efficient transcription; this region contains several potential binding sites for the OCT-type transcription factors. Band shift experiments indicate that human teratocarcinoma cells have a protein that binds to this region, and may be involved in cell type specificity of L1Hs expression. The ORF1 product (p40) in the cytoplasm of teratocarcinoma cells appears to be present in high molecular weight complexes, consistent with the ability of pure p40 synthesized in bacteria to form multimeric structures.

DNA REPLICATION

Studies by the group of Michael Yarmolinsky and Dhruba Chattoraj on the mechanisms by which stably inherited genetic elements replicate and distribute to daughter cells have expanded in new directions. A collaborative effort to characterize a critical control region of the linear plasmid prophage N15 and a compilation and analysis of DNA sequences of the annular plasmid prophage P1 have resulted in the identification of new genes of interest in both plasmids. Physical characterization of chaperone-independent mutant forms of the P1 initiation protein RepA has clarified the nature of the involvement of RepA in copy number control. A preliminary characterization of mutant forms of P1 partition protein ParB that were obtained by selection for dysfunction defectives has revealed the power of this novel technique. It has generated a variety of partition mutants that would be difficult to obtain without tedious screening. A biochemical study of P1 plasmid addiction, previously characterized genetically in this laboratory, has also been initiated with purified plasmid and host proteins.

Recent studies by Michael Lichten and colleagues elucidating the roles that both chromatin and chromosome structure play in determining the sites of meiotic recombination were the subject of a landmark paper published in Science. They showed that double-strand DNA breaks, which initiate meiotic recombination, occur exclusively at sites that display enhanced sensitivity to non-specific nuclease digestion of chromatin *in vitro*, suggesting that accessibility of DNA in chromatin controls where breaks occur. They have mapped the location of double-strand breaks at high resolution at several sites, providing further information about the substrate requirements of the break-forming activity and of the biochemical nature of the breaks themselves. They have also provided evidence, through studies of recombination between dispersed homologous sequences, that chromosome pairing precedes meiotic recombination.

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

PROJECT NUMBER
Z01 CB 00366-23 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Endogenous retroviral elements as indicators of cell function

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

E. L. Kuff	Chief, Biosynthesis Section	LB	NCI
J. Fewell	Microbiologist	LB	NCI
V. Morozov	Visiting Fellow	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.

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Biosynthesis Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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.)

We previously identified a 150 kD heterodimeric protein, designated EBP-80, which stimulated the promoter activity of IAP LTRs in vitro. EBP-80 proved to be identical to Ku, a DNA-binding protein. Ku binds to ends of duplex DNA and, as described by us in previous annual reports, to nicks and larger single strand gaps, stem-loop configurations, and internal regions of strand separation. Ku/EBP-80 helps to regulate transcription of U1 and rRNA in vitro. It is also found as the DNA-binding and activating subunit of a DNA-dependent protein kinase, DNA-PK. We have found that stimulation of DNA-PK by various DNA constructs closely paralleled their affinity for EBP-80. Recently, Ku has been reported to bind to the RNA transcript of the HIV *tar* sequence. The *tar* transcript is thought to assume a stem-loop configuration which is the binding target of the viral *tat* gene product. Using plasmid constructs containing *tar* and other palidromic sequences in sense and antisense orientation, Dr. Morozov is asking whether DNA-PK is activated by binding to the tar RNA and if so, to what level and with what degree of sequence or configurational specificity.

Investigation of the intracellular distribution of EBP-80/Ku has been continued. By immunostaining, Ku is localized in the cytoplasm of sparse cells in culture and moves progressively into the nucleus as cell density increases. We have developed evidence that this redistribution is a response to cell-cell contact. We are currently using antibodies against TGF-α, calherin and the β_1 subunit of integrin to test whether these proteins, which are often involved in cell-cell adhesion, affect the intracellular distribution of Ku. We are also studying molecular changes associated with nuclear transport of Ku and the catalytic subunit of DNA-PK.

Project Description

Major Findings:

A. The DNA-binding protein EBP-80/Ku (Dr. V. Morozov).

Previously we described the isolation of EBP-80 as a transcription factor for *in vitro* promotor activity of IAP LTRs, and the subsequent identification of EBP-80 with Ku, a well-known general DNA-binding protein. EBP-80/Ku is a heterodimeric protein with subunits of 70 and 86 kD, both of which have been cloned and sequenced from human and mouse sources. Ku is known to bind to ends of double-stranded DNA with high affinity, but not to closed circular forms. It has been isolated as the DNA-binding subunit of DNA-PK, a DNA-dependent protein kinase that can phosphorylate a number of important regulatory proteins such as *fos*, *jun*, p53, SV40 T antigen and the C-terminal domain of RNA polymerase II. Ku has also be shown to activate transcription of U1 RNA and rRNA *in vitro*. However, the *in vivo* functions of Ku are not known.

Last year we reported that isolated DNA-PK, in the presence of saturating levels of EBP-80, was activated by a number of DNA configurations other than double-strand ends which we had previously identified as binding sites for EBP-80: nicks and larger single-strand gaps, stem-loop forms, and internal regions of strand separation (bubbles). Dr. Morozov has shown that DNA-PK is activated to about the same extent by all of these perturbations in B-DNA structure. The data extend the range of possible *in vivo* functions of DNA-PK.

During the past year, investigators elsewhere reported that Ku bound to the stem-loop structure predicted for the RNA transcribed from the *tar* sequence of HIV. Binding was assessed by gel-shift experiments. Affinity for the *tar* RNA was said to be about an order of magnitude less than the affinity of Ku for the ends of duplex DNA. Binding was reduced about 50% by mutation in the major loop of the RNA, and nearly completely by mutation affecting the stem. Dr. Mozorov has addressed the question of whether DNA-PK is activated by binding to *tar* RNA. He constructed plasmids containing the *tar* sequence element in both sense and antisense orientations, and one containing a random DNA sequence of total overall length. Results thus far show that the sense form of the *tar* RNA is relatively ineffective in activating DNA-PK compared to a 64 bp linear duplex oligonucleotide assayed as a standard. Whereas activation was maximal with the 64 mer at 7.5 nM, *tar* RNA had stimulated activity to only 20% of the maximum at 200 nM. Thus, on a molar basis, *tar* RNA appeared to have less than 1% of the stimulatory activity of the DNA standard [(7.5/200) x (20/100)]. Further study of this and the other constructs is in progress.

B. Growth-related changes in the intracellular distribution of Ku and DNA-PK (Mr. J. Fewell).

In last year's report, we described observations showing that Ku, as detected by immunofluorescent (IMF) staining with both polyclonal and monoclonal antibodies, underwent a marked charge in its intracellular distribution as cells in culture progressed from sparse conditions to confluence. Immunoreactive antigen, localized primarily to the cytoplasm in sparse cells, became progressively concentrated in the nuclei with increasing cell density until at confluence, nuclear IMF was greatly predominant, with little or no cytoplasmic reactivity. This sequence of events was seen in 4 human transformed cell lines and in cultured human primary keratinocytes. The past year has been spent in repeating a number of the experiments to obtain high-quality illustrative data for publication. In addition, Mr. Fewell has made two other new observations with respect to Ku. First, he has found that the antibodies against both Ku subunits react in IMF with a juxtanuclear body that resembles the centrosome in position and size. If the observation can be confirmed by electron immunomicroscopy, it will represent the first time that a DNA-binding protein has been localized to this organelle. Second,

using a polyclonal rabbit antibody against the 300 kD catalytic subunit of DNA-PK, he has shown that this protein shows a change in intracellular distribution with increasing cell density that is similar to that seen with EBP-80/Ku. We are currently testing the possibility that Ku, either alone or as part of the holoenzyeme DNA-PK, responds to signals generated by cell-cell contact and perhaps itself has a role in the signal transduction process.

C. Interaction between EBP-80 and IAP LTRs.

Nothing was done on this subject during the reporting year.

D. Patterns of IAP LTR hypomethylation in primary and transplanted plasmacytomas and B-cell lymphomas of BALB/c mice.

See Annual Report of Dr. Kira Lueders.

Publications:

Kuff EF, Mietz JA. Analysis of DNA restriction enzyme digests by 2D electrophoresis in agarose gels. In: A. Harwood, ed. Methods in molecular biology. England: Humana, 1994;177-86.

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 1993;268:10546-52.

Lueders KK, Fewell JW, Morozov VE, Kuff EL. Selective expression of intracisternal A-particle genes in established mouse plasmacytomas, Molec Cell Biol 1993;13:7439-46.

Morozov VE, Falzon M, Anderson CW, Kuff EL. DNA-dependent protein kinase is activated by nicks and larger single-stranded gaps, J Biol Chem 1994;269:16684-8.

Lueders KK, Kuff EL. Intracisternal A-particle (IAP) genes show similar patterns of hypomethylation in established and primary mouse plasmacytomas, Current Topics in Microbiology and Immunology, in press.

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

PROJECT NUMBER
Z01 CB 00945-21 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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
R. Kim	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 STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	3.25	1.25

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.)

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. We have shown that circulating insulin-like growth factor binding proteins (IGFBPs) 1 and 2 induced during scurvy and fasting inhibit these functions and DNA synthesis in cultured connective tissue cells and inhibition is reversed by insulin-like growth factor (IGF)-I. These IGFBPs are induced prior to or concomitant with the decrease in collagen mRNA concentrations in bone, skin and cartilage of fasted and scorbutic guinea pigs, which suggests that they also function as inhibitors of collagen synthesis in vivo. Our present studies examined the possibility that IGFBPs expressed in tissues also may contribute to regulation of IGF-I activity and consequently to regulation of collagen gene expression. There was no expression of IGF-I or IGFBP-1 mRNAs in any of the connective tissues of fasted guinea pigs although IGFBP-1 itself was present at levels paralleling serum concentrations, indicating it regulates exclusively by an endocrine mechanism. IGFBP 2 mRNA was significantly expressed only in bone while the protein was present in all tissue extracts. Thus in bone, collagen gene expression could be regulated by IGF-I and its binding proteins through a combination of endocrine and autocrine/paracrine mechanisms during fasting.

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 vitamin C, insulin-like growth factors (IGFs) and IGF-binding proteins in this regulation.

Major Findings:

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).

Using antibodies to purified IGFBPs 1 and 2 we proved that inhibition was caused by the IGFBPs, although IGFBP-1 was more potent. We also showed that the circulating IGFBPs were induced prior to the decrease in collagen mRNAs in bone, skin and cartilage in both fasting and vitamin C deficiency, although the pattern of induction of IGFBP-1 paralleled that of the inhibitor of IGF-I action. These results suggested that circulating IGFBPs 1 and 2 could also inhibit collagen gene expression *in vivo*. The present studies were undertaken to examine the possibility for local regulation of IGF-I action by IGFBPs. To accomplish this, the levels of mRNAs for these factors were measured by slot-blot hybridization and the protein levels of the IGFBPs in tissues were measured by ligand blotting after fasting guinea pigs for 10-96 h.

B. Expression of IGF and IGFBP mRNAs in Connective Tissues

1. IGF-I mRNA was not expressed in any of the connective tissues in normal or fasted animals. IGF-II, which has a lower affinity for the IGF-I receptor, was expressed in bone and cartilage.

2. IGFBP-1 mRNA also was not expressed in any of the connective tissues but the protein was found in tissue extracts of fasted animals and the concentrations paralleled those in the circulation.

3. IGFBP-2 mRNA was significantly expressed only in bone, but there was relatively little induction during fasting. The protein, however, was found in tissue extracts from all the connective tissues.

C. Conclusions

Regulation of collagen gene expression mediated through IGF-I and IGFBPs 1 and 2 must occur through strictly endocrine mechanisms in skin and cartilage although in bone there is a possibility for additional autocrine or paracrine regulation by IGFBP-2.

Publications:

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

PROJECT NUMBER
Z01 CB 05202-27 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

O.W. McBride	Chief, Cellular Regulation Section	LB	NCI
Keming Lin	Visiting Fellow	LB	NCI
H.-F. Yi	Visiting Fellow	LB	NCI
M.G. Wang	Guest Researcher	LB	NCI
J. Clark	Laboratory Worker	LB	NCI

COOPERATING UNITS (if any) NIAMSD: P. Steinert, S.J. Bale; NCI: K.Kelly, S.Rosenberg, F. Gonzalez, J. Battey, B. Mock, C. Wu; NIMH: S.J. Lolait, A.M. O'Carroll; NIDR: L. Fisher, F. Luyten; NIAID: K.T. Jeang, C. Kozak, E. Max; A. Arnold (Harvard); M. Nussmeier (U. Chicago); R. Pirtle (U. Texas); E.J. O'Keefe (U. North Carolina); G. Serrero (Lake Placid, NY); E. Carafoli (Zurich)

LAB/BRANCH
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SECTION
Cellular Regulation Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.7	2.2	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.)

During the past year, about two dozen additional cloned genes have been chromosomally localized in collaboration with investigators at NIH and elsewhere by three complementary methods including Southern blotting of a panel of human/rodent somatic cell hybrid DNAs, fluorescence in situ hybridization, and genetic linkage analysis in CEPH pedigrees. A somatostatin receptor (SSTR4) cloned by A.M. O'Carroll has been localized to 16p13.3 in close proximity to the major locus for polycystic kidney disease, and it is currently a candidate gene for this disease. Efforts are in progress to obtain DNAs from individuals (PKD1) and their unaffected relatives; DNA sequencing will permit identification of mutations in individuals with the disease or conversely the exclusion of involvement of this gene in PKD1. Candidate genes for several hereditary skin diseases including three transglutaminases have been further localized by FISH and genetic linkage analysis (TGM1) to allow evaluation of whether any of these genes are involved in these diseases. Improved methods have been developed to permit isolation of STRPs (short tandem repeat polymorphisms) at candidate gene loci.

Project Description

Objectives:

1) Human chromosomal mapping of protooncogenes and genes involved in DNA synthesis, carcinogen metabolism, and regulation of cell proliferation and gene expression, and understanding role of these genes in human neoplasia, 2) mapping the genes for hereditary cancer predisposition syndromes, 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 is used for chromosomal mapping of cloned human genes in collaboration with investigators at NIH and elsewhere. Several genes localized during the past year using this panel include a vasopressin receptor (V1a) (12p12.1-qter) and somatostatin receptor (SSTR4)(16p13.3) with S. Lolait & A.M. O'Carroll; two bone morphogenetic protein genes (2q11-qter & 20) with F. Luyten; TAR RNA-binding protein gene (12p12.1-q13.1) and processed pseudogene (8q22-qter) with K.T. Jeang & C. Kozak; intermediate filament associated protein gene, IFAP (chr. 20) and sublocalization of TGM2 & TGM3 to 20q11.2 with P. Steinert; Gαq protein gene and pseudogene to chr. 9 & 2q11-qter with J. Battey; heat shock factor 1 (HSF1) (8q24) with C. Wu; diphosphopyridine dehydrogenase (DPD) (1p22-q21) & carboxyesterase (CE1)(chr. 16) with F. Gonzalez; drebin (chr. 5) with L. Fisher; CYP b561 (17q11-qter) with M. Srivistava; J chain pseudogene (8q13-q21) with EE Max; transcriptional activator CHK (chr 10) with B. Mock (NCI) & K. Marcu (Stony Brook); four members of nuclear receptor superfamily of transcription factors (PPARG, 3p25; HOR-1, 15q21; HOR-2, 11q13; TR-2, 12p12.1-qter) with M. Nussmeier; adipose differentiation-related protein gene (chr 9) and pseudogene (1p12-q32) with G. Serrero; gene for 37 kDa protein h32 (chr 19) with E. O'Keefe; Na^{+}-Ca^{++} exchanger genes NCX1 & related gene NCXL1 (2p21-23 & 14q24.3) with E. Carafoli (Zurich).

Somatic cell hybrids containing human chromosomes with specific breaks and translocations have been used by us to regionally localize many genes on chromosomes, and specific assignments were also obtained by both fluorescence in situ hybridization (FISH) and genetic linkage analysis. Both TGM2 and TGM3 were assigned to the same band location (i.e., 20q11.2) by FISH, and even more precise determination of the distance between these two tissue-specific transaminase genes awaits results of linkage analysis. Both are candidate genes in several hereditary skin diseases, and the development of highly polymorphic markers at these sites is important for this purpose. PMCA3 has been localized to Xq28 both by FISH and by linkage analysis.

Several interesting findings include localization of the somatostatin receptor, SSTR4, to 16p13.3. A single strand conformational polymorphism (SSCP) was detected within the 3'UTR of this gene and used for fine regional localization. The gene maps to precisely the same small interval (< 2-3 megabases) containing the most common locus for polycystic kidney disease (PKD1). More refined

linkage analysis is currently being completed and an effort is in progress to obtain DNAs from patients with PKD1 for DNA sequencing to determine whether a defect in SSTR4 could be involved in this disease.

HSF1 cloned by Carl Wu and colleagues appears to be located near the telomere of 8q. Consistent with this fact was the identification of a VNTR (variable number of tandem repeats) within intron 10 of the gene, and this polymorphism was used for linkage analysis with other loci on chromosome 8. Unfortunately, it has not been possible to amplify this intron by PCR, and this appears to be related to a very high GC content of this region. PCR amplification will permit DNA sequencing of this intron and development of a more sensitive and convenient assay for length polymorphisms. A weakly hybridizing nonsyntenic locus is also detected by Southern blotting with an HSF1 cDNA probe, and this may represent an anticipated second heat shock locus, HSF2.

A human cDNA for one member of the family of alpha subunits of human G proteins, Gαq, was recently cloned by J. Battey, and it has been used for gene mapping. Preliminary studies by Battey and A. Spiegel suggested that a retropseudogene was also present. A 700 bp fragment from the 3'UTR (about 2 kb distal to coding sequences) was used as probe for Southern blotting, and the hybridizing human sequence did not cosegregate with any human chromosome. This occurred because sequences on two different human chromosomes were identified by this probe, and the EcoRI and HindIII fragments at both loci were indistinguishable in length. Surprisingly, this region is highly conserved in rodents suggesting that it may be important for gene regulation, mRNA stability, or some other function. Use of probes from other portions of the cDNA permitted assignment of these sequences to human chromosomes 2q and 9. The functional gene is probably on human chr. 9 since Gαq has previously been assigned to mouse chromosome 19, and human chromosome 9 (but not chromosome 2) has homology with this mouse chromosome. Further studies are required to confirm this assumption, and to sublocalize both sequences on these chromosomes.

While mapping of human genes for two bone morphogenetic proteins was in progress, the cloning and mapping of the GDF5 gene in mouse was reported by Storm et al. (Nature 368:639, 1994). This gene is the mouse homologue of one of the human genes (CDMP4). Moreover, a mutation at various sites in the GDF5 gene was shown to cause brachypodism in mice. The location of this gene on human chromosome 20 is homologous to the locus on mouse chromosome 2 and confirms our assignment. It will be interesting to determine whether similar genetic abnormalities in humans involve mutations in this gene. Linkage analysis is being used to further localize both of these human genes.

The TAR RNA-binding protein is important in transcriptional regulation of HIV-1. Localization of this gene to the same chromosomal location previously reported to be important for optimal interactions between Tat and TAR in rodent cells and HIV replication is not unexpected.

High density linkage maps for most of the human genome have become available over the past two years based primarily on identification of short tandem repeat polymorphisms (STRPs) by J. Weissenbach, J. Weber, and others. Genetic linkage analysis in the large CEPH pedigrees in combination with these maps is now being used for high resolution mapping of most cloned genes in our laboratory. During

the past year, this has been used in mapping TGM1, NCX genes, BSP and osteopontin, the thrombospondin receptor CD36, SSTR4, ATP2B3, PPARγ, HOR-1, HOR-2, a J-protein retropseudogene, and calmodulin. STRPs at these loci have been most useful for this purpose but SSCPs and RFLPs have also been used when no STRPs were available.

Isolation and identification of genes for human diseases in which only a few small families are available has assumed increasing importance. All candidate genes are evaluated for presence of RFLPs in at least ten individuals with 12 different restriction endonucleases. All DNA sequences are also inspected for the presence of STRPs. More recently, a more direct approach has been used to identify short tandem repeat sequences. Aliquots of DNA from all available recombinant genomic clones (phage, cosmids, or plasmids) are blotted onto nylon membranes after digestion with restriction enzymes and agarose gel electropheresis. These blots are hybridizeed with ^{32}P-labeled oligonucleotide probes consisting of specific di-, tri-, and tetra-nucleotide repeat sequences. Short (i.e. < 5 kb) single and double digested fragments containing STRP sequences are purified by gel electrophoresis, subcloned into plasmid vectors, and the plasmid DNA minipreps are tested for presence of the appropriate repeat. The plasmid DNA is linearized within the vector and used as DNA template for PCR amplification using the oligonucleotide repeat sequence (and the complementary sequence in separate reactions) and one of a pair of universal primers of vector sequence flanking the multiple cloning site. Usually the STRP can be amplified in at least one orientation, and directionally subcloned into plasmid after digestion with appropriate restriction enzymes. Small recombinant plasmid DNA preps are used for dideoxy DNA sequencing using a universal primer flanking the cloned STRP. After two short sequencing reactions, specific oligonucleotide primers flanking the STRP can be synthesized and polymorphism at the site can be evaluated by PCR amplification of a few DNAs. Preliminary results indicate that this method will probably be useful for identifying STRPS flanking most of these candidate genes. An $(AAC)_N$ repeat has been isolated from an intron in ATP2B1 on chromosome 12 by this method and it is currently undergoing testing in the CEPH families. SSCPs are used when no STRPs can be identified.

Publications:

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

PROJECT NUMBER
Z01 CB 05203-26 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Immunochemical Purification and Characterization of Immunocytes and Components

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

M.G. Mage	Research Biologist	LB NCI
L.L. McHugh	Biologist	LB NCI
L. Lee	IRTA Fellow	LB NCI
J. Zhu	Visiting Fellow	LB NCI

COOPERATING UNITS (if any)
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Laboratory of Biochemistry, DCBDC

SECTION
Biosynthesis Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.00	4.00	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors B
☐ (a2) Interviews AIDS research: 50%

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

Our laboratory's current interest is in development of recombinant single chain MHC molecules in order to study their cell biology, immune function, and potential use as vaccine components. This is an outgrowth of our 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) and as potential immunogens.

Class I MHC molecules bind peptides from endogenously synthesized proteins and display them on the surface of infected cells as an MHC-peptide complex. The recognition of this complex by the specific receptors of $CD8^+$ T cells is an important component of antiviral immune responses, including resistance to HIV. Our recombinant single chain class I MHC molecules, unlike the wild type two chain molecules, have a non-dissociable $\beta 2$ microglobulin domain.

During this year we have studied the expression in mammalian and bacterial cells of single chain MHC genes that were constructed in the previous year, and have begun the use of soluble single chain MHC molecules in construction of macromolecular conjugates for immunization. We have found that recombinant genes for single chain $H\text{-}2L^d$, and for the human class I MHC molecule HLA-A2.1 can be successfully expressed in mammalian cells. This generalizes our previous results and extends to three the number of different single chain molecules that we have produced. We have also expressed a class I MHC molecule as a covalently linked heterotrimer of $\beta 2m$, heavy chain, and antigenic peptide, and are currently working on improving its configuration.

Project Description

Major findings:

A. We have obtained expression of a recombinant soluble single chain H-2L^d gene in mammalian cells. These cells secrete a soluble single chain H-2L^d protein. This finding extends and generalizes our previous successful expression of H-2D^d as a recombinant single chain MHC molecule. Single chain H-2L^d is being harvested from culture supernatants in order to affinity purify it and attempt co-crystallization with a soluble T cell receptor, in order to better understand the nature of the interaction between these two molecules. Interestingly, accumulation of secreted protein is better at 27^o than at 37^o, a finding that is also true for wild-type two chain H-2L^d, and for single chain and wild type H-2D^d molecules. Accumulation is the net result of secretion rate and thermal degradation. Although synthesis may be slower at the lower temperature, more molecules may be stabilized by peptides in the endoplasmic reticulum at this temperature. At 27^o there could thus be both an increased secretion rate and less thermal degradation than at 37^o.

B. We have also obtained expression in mammalian cells, including human cells, of a recombinant human single chain HLA-A2.1 gene that was constructed last year. Cells expressing the recombinant protein on their surface could be pulsed with an appropriate peptide and lysed by cytotoxic T cells, showing that the recombinant HLA-A2.1 molecule was biologically active. This gives us three examples of successful expression of recombinant single chain MHC molecules.

C. If an antigenic peptide is covalently attached to a recombinant MHC molecule, the entire heterotrimer of ß2m, heavy chain, and peptide could be synthesized as a single nondissociable unit. This would have advantages as an immunogen, compared to molecules with a dissociable antigenic peptide. We have obtained expression of a soluble single chain H-2D^d molecule with a covalently attached antigenic peptide. The molecule did not react with an alpha 2 domain-specific mAb, nor did it present its peptide to the T cell hybridoma. This indicates that the peptide binding groove was either incorrectly folded or that the antigenic peptide, in the wrong position, blocked access of the mAb. The molecule could bind and present free peptide, showing that it was correctly folded, but unable to bind the covalently attached peptide in its binding groove. In order to get better access to the binding groove for the covalently attached peptide, we are doing site directed mutagenesis to create a better path for the spacer that links the peptide to the rest of the molecule.

D. We have found that our method for loading soluble MHC molecules with peptides by means of a brief exposure to high pH can also be used with cell surface MHC molecules. This provides a convenient way to pulse cell surface MHC molecules with peptides for presentation to T cells.

E. In order to have large amounts of material for structural studies and for experimental immunizations, we have developed a bacterial expression system for the soluble single chain H-2D^d, in collaboration with David Margulies and Shozo Sakuma (LI, NIAID). These molecules can be refolded after denaturing in guanidine·HCl. Correct conformation is shown by their ability to present antigenic peptide to the appropriate T hybridoma.

F. We would like to know if an experimental vaccine that provides costimulation to T cells would elicit an improved immune response. In collaboration with Jay Berzofsky and Graham Leggett (MET, DCBDC, NCI) we are working on the approach of conjugating both peptide-pulsed single chain MHC molecules and costimulatory molecules (such as anti CD28) to the same macromolecular carrier. To

correctly orient the MHC component of such a conjugate, we have prepared a bimolecular conjugate of soluble single chain H-2D^d bound to an antibody to its Q10 tail (at the C terminal of the alpha 3 domain, far from the peptide binding site). We plan to bind this conjugate, together with anti-CD28, to a carrier molecule and test the resultant heteroconjugate as an immunogen.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05231-20 LB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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,

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
Z.-H. Gao	IRTA Fellow	LB	NCI
S. Choi	General Fellow	LB	NCI

COOPERATING UNITS (if any) Dr. A. Bax, NIDDK; Dr. S. Schreiber, Harvard Univ.; Dr. S. Burakoff, Dana-Farber Cancer Inst., Boston; Dr. Ching Kung, U. of Wisconsin; Dr. O.W. McBride, NCI; Dr. Eva Mezey, NINDB; Dr. J. Anglister, NIDDK (Weizmann Inst., Israel); Dr. L. Pinna (U. of Padova, Italy; Dr. W. Horrocks, Jr., PSU.

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Protein Biochemistry Section

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.75	5.25	1.50

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.)

Work in this group 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. The ultimate goals are to elucidate the structure of the calcineurin/calmodulin complex and to determine the molecular basis for the inhibition of calcineurin by the two immunosuppressive drugs, FK506 and cyslosporin. During the past year Mr. Hao Ren has succeeded in expressing the two subunits of calcineurin in E. coli. The secondary structure of the regulatory subunit, calcineurin B, has been determined in collaboration with Drs. Adrian Bax and Jacob Anglister (NIDDK). An enzymatically active enzyme was reconstituted from the two recombinant subunits and is being used to crystallize and determine the structure of calcineurin complexed with calmodulin. The recombinant proteins modified by site directed mutagenesis are also being used by Drs. Ann Stump and Igor Myagkikh to identify the interaction domains of calcineurin with the immunosuppressive drug, FK506, and the active site of the enzyme.

Project Description

Objectives:

To study the functional roles of protein-protein interactions in the regulation of cellular processes. The system under investigation is the Ca^{2+}-dependent regulation of enzymes mediated by calmodulin. Emphasis is on the mechanism of the regulation of the Ca^{2+}-dependent stimulation of the protein phosphatase, calcineurin, by calmodulin. These studies are undertaken to elucidate the role of protein phosphorylation in signal transduction.

Calmodulin and Calcium Regulation of Cellular Activity

A. Interaction of Calmodulin with Target Proteins and Peptides

The mechanism of calmodulin activation of most calmodulin-regulated enzymes involves the displacement of an autoinhibitory domain by binding of the Ca^{2+}-calmodulin complex to a calmodulin-binding domain. One of our goals is to identify the differences in the interaction of calmodulin with its targets that insure the temporal and topological coordination of cellular responses to the Ca^{2+} signal. Conformational changes accompanying the sequential occupancy of the four Ca^{2+} sites of calmodulin enable calmodulin to interact with and activate target enzymes. The identification of these conformational changes requires the elucidation of the structure of Ca^{2+}-free and partially liganded calmodulin. Determination of the structure of Ca^{2+}-free calmodulin is presently being carried out in collaboration with Adriaan Bax (NIDDK). Calmodulin mutants in the amino-terminal half of the molecule isolated in Ching Kung's laboratory (University of Wisconsin) are being used to obtain additional evidence for specific roles of the amino and carboxyl halves of calmodulin. These mutants were shown to activate calcineurin only partially. The crystal structure determination and the analysis of the Ca^{2+}-binding and enzymatic properties of these mutants were initiated to identify the structural defect leading to the phenomenon of partial activation (Igor Myagkikh in collaboration with Ching Kung and M. Sundaralingam from Ohio State University).

B. Calcineurin Structure Function Relationships

We reported last year the large scale expression of the regulatory subunit of calcineurin, Calcineurin B, in *E. coli* by Ren Hao. The secondary structure of calcineurin B, determined in collaboration with Drs. Adriaan Bax and Jacob Anglister (NIDDK), confirmed that calcineurin B is a member of the "EF-hand" Ca^{2+}-modulated proteins. Despite their strong structural similarity, calmodulin and calcineurin B are functionally distinct. Calcineurin B cannot replace calmodulin in the activation of the enzyme and calmodulin cannot substitute for calcineurin B in the reconstitution of enzymatically active calcineurin. The dependence of calcineurin on Ca^{2+} for activity is the result of the concerted action of two different Ca^{2+}-regulated proteins. Calmodulin and calcineurin B activate calcineurin by different and complementary mechanisms. Calmodulin increases the turnover rate of the enzyme and modulates its response to Ca^{2+} transients. Ca^{2+} binding to calcineurin B decreases the Km of the enzyme for its substrate and facilitates its activation by calmodulin.

Calmodulin and calcineurin B specifically interact with distinct domains on calcineurin A. The calcineurin B-binding domain of calcineurin A has been identified. It is located between residues 349 and 385 of calcineurin Aß. A synthetic peptide whose sequence corresponds to the sequence of this calcineurin B-binding domain binds to calcineurin B with high affinity. The binding is facilitated by

but does not require Ca^{2+}. This peptide will be used in collaboration with Adriaan Bax and Jacob Anglister (NIDDK) to determine the solution structure of calcineurin B bound to calcineurin A.

Large scale expression of the catalytic subunit of calcineurin, calcineurin A, in *E. coli*, has now been achieved by Ren Hao. Using the two recombinant proteins, Ren Hao was able to reconstitute an enzymatically active enzyme suitable for crystallization studies. Site directed mutagenesis of recombinant proteins is presently used by Ann Stump and Igor Myagkikh to identify the residues important for catalytic activity and interaction with the immunosuppressive drug, FK506.

C. Physiological Roles of Calcineurin in Cellular Signaling

Two different approaches are being followed to identify the physiological roles of calcineurin. The presence on the calcineurin molecule of a recognition site for FKBP and cyclophillin complexed with their respective immunosuppressive drugs led us to postulate the presence of naturally occurring cofactors acting as endogenous ligands of the immunophillins. Novel small molecular factor(s) regulating calcineurin activity, previously detected by Paul Stemmer in crude brain extracts, are being purified and characterized by Xutong Wang.

A collaborative project with Drs. Hursh and Mortin in our laboratory was initiated to use Drosophila genetics to identify the putative roles of calcineurin in other tissues. Two different clones for calcineurin A and a unique calcineurin B clone have been isolated from cDNA *Drosophila melanogaster* libraries and sequenced in our laboratory. The three calcineurin genes have been localized on the *Drosophila* chromosomes by Mark Mortin and Tammy Jones. Genomic clones have recently been isolated and will be used to rescue known mutations in these loci, as well as new calcineurin mutants generated by Dr. Hursh, in order to identify the regulatory roles of calcineurin in flies.

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

PROJECT NUMBER
Z01 CB 05244-17 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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J. McMillan	Visiting Fellow	LB	NCI
A. Clements	IRTA	LB	NCI
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COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.65	4.4	0.25

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☐ (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.)

Our aim continues to be the understanding of the mechanism and regulation of transposition of LINE-1 elements in the human genome (L1Hs). To this end, work has continued on the cis-regulatory elements controlling cell-type specific transcription of L1Hs in human teratocarcinoma cells. In the past year, attention focused on a region between residues 60 and 100 of the 900 bp long 5' UTR. Deletion derivatives of the 5' UTR incorporated into a reporter gene construct and transfected into human teratocarcinoma cells indicate that the region between residue 70 and 100 contains sequences important for efficient transcription and this region contains several potential binding sites for the Oct-type transcription factors. Band shift experiments with radioactive oligonucleotides from this region demonstrated that the ubiquitous factor Oct-1 interacts with a probe representing base pairs 80-100 and that one of the potential OCT sites is important for this binding. However, when sequences from 60 to 80 are included in the probe (i.e., 60-100) the Oct-1 band markedly diminishes and a second band, involving unknown protein(s) appears; this band is much less evident with HeLa cell nuclear extracts or those obtained from just confluent NTera2D1 cells, compared to those from embryonal carcinoma phenotype NTera2D1 cells or 2102EP teratocarcinoma cells. Thus, this band may be responsible, at least in part, for cell-type specific expression of L1Hs.

No evidence for the occurrence of p40 in a particulate structure in teratocarcinoma cells could be obtained. However, the 40 kDa protein fractionates within a range of high molecular weights, consistent with the ability of pure p40 synthesized in bacteria to form homodimers and higher multimeric structures.

Project Description

Objectives

The LINE-1 element (L1Hs) is the only transposable element known to be currently active in the human genome (Alu elements, which do not carry any genes required for their own transposition, are not here included among transposable elements). Several of the approximately 3500 full length L1Hs elements in the genome appear to be actively transposing; they have been detected by virtue of their mutagenic transposition into genes and consequent disease. Our aim is to understand the mechanism and regulation of L1Hs transcription and translation and the nature of L1Hs encoded proteins in order to elucidate the mechanism and control of transposition. Much of our work during this last year has been in two areas: 1) the *cis*-acting transcriptional regulatory motifs in the 900 bp L1Hs 5'UTR (untranslated region) which we previously demonstrated contains all the sequences required to promote transcription in a cell-specific manner and 2) continued investigation of p40, the polypeptide encoded by the first of the two open reading frames in full length, active L1Hs elements.

Major Findings:

Regulation of L1Hs Transcription

Previous work indicated that significant *cis*-acting regulatory motifs are spread throughout the first 600 bp of the 5' UTR of transcriptionally active L1Hs elements and that base pairs 1-100 are especially important. Thus, deletion of base pairs 1-101 from a reporter gene construct in which *lac*Z was fused in frame after the first 15 codons of ORF1, reduced β-galactosidase activity in transfected cells to 0.3 percent of that obtained with the complete 5' UTR. Deletion of base pairs 1-18 alone reduced β-galactosidase activity to 20 percent of the control and this was shown to correlate with the presence, in this region, of a binding site for the ubiquitous transcriptional regulatory protein, YY1. While YY1 may be important for optimal transcription of L1Hs, it is unlikely to account for the cell-type specificity of transcription (in teratocarcinoma and certain other tumor cells). Previous (unpublished) experiments showed that deletion of base pairs 1 through 68 or 87 resulted in expression levels of 10 or 1 percent of control, respectively. Moreover, 95 percent of activity is lost upon deletion of base pairs 71 through 99 and other workers (Sakaki and coworkers) observed a DNA footprint around residues 71-89 with HeLa cell extracts. In addition, comparison of β-galactosidase expression from constructs lacking base pairs 98 to 390 or 126 to 385 indicate that the segment between 98 and 126 also contains important regulatory sequences. Indeed, we have now found that if all the 5' UTR sequences 3' to residue 157 are deleted from the 5' UTR, activity is on the order of 20 percent of that obtained with the full 900 bp 5' UTR. Inspection of the L1Hs sequence revealed the presence of two, tandemly arranged 8 base pair sequences (residues 78 to 93) that are similar to the canonical binding site for the OTF (Oct) family of POU domain transcriptional regulatory proteins or transacting factors (TF).

To investigate the potential role of these TFs in L1Hs expression, an extensive series of band shift experiments (EMSA) was carried out using nuclear extracts from teratocarcinoma and HeLa cells and labeled oligonucleotides representing sequences between residues 60 and 120 of the 5' UTR as well as unlabeled oligonucleotides as competitors. With all cell extracts, multiple labeled bands appear when the ^{32}P-probe represents base pairs 80-100, 80-120, or 60-100. The bands are the same with probes 80-100 and 80-120; the band with the lowest mobility, A, predominates with extracts of just confluent NTera2D1 cells and of HeLa cells but not of the teratocarcinoma line 2102EP or piled up NTera2D1 cells (embryonal carcinoma phenotype). Band A is competed by unlabeled 80-100 but not if the residues 84 to 89 are mutated, and the band is shifted or ablated by antibody to the ubiquitous TF, Oct-

1. If sequences from 60 to 80 are included in the labeled probe, i.e., 60-100, band A does not appear (or is very weak) and band B, which is a minor or variable band with 80-120, becomes the dominant low mobility band using extracts from both just confluent and piled up NTera2D1 cells. Similarly, band B, which is not seen with 2102EP nuclear extracts with probes that start at residue 80, is abundant with probes that start at residue 60. Band B, like band A, is not competed by oligonucleotides in which residues 85-89 are mutated, suggesting that an Oct-type TF may be involved in band B formation; band B is unaffected by antibody to either Oct-1 or Oct-2. Thus, in the presence of the segment from 60-80 binding to Oct-1 is suppressed and another protein factor(s) successfully competes for the oligonucleotide. Besides the ubiquitous Oct-1 and the B cell-type specific Oct-2, an extensive family of mammalian Oct proteins that are expressed in cell-type specific manner has been described. In particular, Oct-3/4, Oct-6, and Oct-11 appear to be present in early embryonic cells. Because the protein(s) responsible for formation of band B are not abundant in HeLa cell nuclear extracts, it may reflect binding with one of these known Oct factors and be responsible, at least in part, for the specific expression of L1Hs in teratocarcinoma cells. Formation of a complex between this protein and the 5' UTR of L1Hs must involve the OCT binding site as well as other sequences between residues 65 and 80.

Studies on p40

The polypeptides predicted by the 5' ORFs (ORF1) of mammalian LINE-1 elements have little homology to any sequences in the available data banks. Thus, in spite of the location of these ORF1s in the position occupied by the gag-like ORFs in other retrotransposons, they do not encode gag-like proteins. Moreover, while all the known ORF1s encode proteins of similar size (between 338 and 379 residues) and their carboxy terminal amino acid sequences are significantly conserved, their amino terminal segments are distinctive from one mammalian order to another. The ORF1 polypeptide of L1Hs, p40 (338 residues), which we previously demonstrated occurs in the cytoplasm of human teratocarcinoma cells, has a potential leucine zipper structure (residues 90 to 131). We have examined the characteristics of this endogenous p40 and found that it fractionates (in the absence of divalent cations) in a variable manner including as an apparently heavy complex of at least 2 million Da and in lighter fractions as well. If cell extracts are treated with RNaseA, the apparent size of most of the p40 shifts to lighter fractions. It appears that in the extracts p40 tends to stick to ribosomes (and some may represent molecules just completing translation). p40 as well as several derivatives carrying deletions in different parts of the molecule were synthesized in *E. coli* These polypeptides can form homomultimers. The leucine zipper region (residues 90-131) appears to be essential for multimerization while other portions of the molecule facilitate the process. For example, under oxidizing conditions, the cysteine residues (all within the first 111 amino acids) can form intramolecular disulfide bonds that stabilize multimers. Under reducing conditions, multimers also form as long as the leucine zipper motif is intact in the polypeptide. The data suggest that residues 93 through 163, which contains the leucine zipper motif, can itself form dimers while the addition of residues from the amino or carboxy terminus of the p40 polypeptide permit the stabilization of higher multimers.

Future Plans

During the coming year we will continue to try to identify the protein(s) responsible for the formation of band B in the band shift experiments. Antibodies to known Oct proteins will be tested, insofar as they are available, to examine whether band B is associated with already identified factors. If it appears that the proteins are not already known, an attempt will be made to clone cDNA for this protein(s) from already existing NTera2D1 cDNA library. We will also identify those sequences between 60 and 80 that are responsible for band B formation. In addition, experiments described in

last year's report on the regulation of L1Hs translation and the expression of ORF 2 proteins, which have continued through this year but without major new findings, will continue as will experiments designed to understand more about the role of p40. Our emphasis on the biochemistry of L1Hs expression will continue.

Publications

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

PROJECT NUMBER
Z01 CB 05258-15 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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I. Karsch-Mizrachi	Visiting Scientist	LB	NCI
D. Hursh	Sr. Staff Fellow	LB	NCI
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COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.53	6.33	0.20

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☐ (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 role of the basic-helix-loop-helix myogenic factors in the process of cell commitment and differentiation during myogenesis, analyzing primary cultures of chick embryonic breast muscle and, more recently for genetic purposes, larval muscle formation in Drosophila. Vertebrates express four different myogenic factors in a developmentally regulated pattern whereas Drosophila has only a single factor. Although these myogenic factors can bind DNA as homodimers, the preferred DNA-binding complex is the heterodimer involving one of the ubiquitous E-related proteins found in both vertebrates and Drosophila. These factors play a dual role for they are involved in myogenic cell commitment and in the activation of muscle-specific genes during myogenesis. Since these factors must dimerize in order to bind DNA yet each can homodimerize, heterodimerize with four E-related proteins and three Id-related negative regulators, dimerization specificity is thought to play a crucial regulatory role in myogenic factor function. We have experimentally shown by comparison with the Drosophila factor that the dimerization specificity within the vertebrate factors is determined, to a large extent, by the nonconserved nonhydrophobic residues in the HLH domain. An analysis of the charge interactions in the HLH domain has allowed us to predict preferred heterodimer combinations and to design a better homodimerizing analog of avian MyoD. This was confirmed experimentally both in vitro and in vivo. This analysis also helped us to identify a partner protein for Drosophila MyoD and to predict that Drosophila MyoD could potentially rescue a C. elegans MyoD null mutant. We have also shown that a unique phosphoserine(s) plays a regulatory role in vertebrate MyoD homodimer formation. The identification of this site(s) will help to identify the target kinase(s) and may reveal a growth factor regulated pathway involved in myogenic factor function. Preliminary analysis indicates that Drosophila MyoD homodimer formation is also regulated by phosphorylation. Promoter analysis of the avian MyoD gene (CMD1) has revealed that auto regulation and cross activation of this gene by MyoD and the other myogenic factors involves an indirect pathway that is E-box and MEF-2 independent. Our recent efforts with the genetic and molecular analysis of the Drosophila MyoD gene will help us to identify genes in the fly's myogenic pathway that will potentially lead to the identification of the related genes in vertebrates.

Project Description

Major Findings:

Studies on Vertebrate Myogenesis

1. Structural and functional characterization of the avian MyoD gene, CMD1.

Members of the MyoD family of gene regulatory proteins (MyoD, myogenin, myf5, and MRF4) have all been shown to regulate not only the transcription of numerous muscle-specific genes but also to positively auto regulate and cross activate each other's transcription. In the case of the muscle-specific genes, this transcriptional regulation can often be correlated with the presence of a DNA consensus in the regulatory region, CANNTG, known as an E-box. Little is known about the regulatory interactions of the myogenic factors themselves; however, these interactions are thought to be important for the activation and maintenance of the muscle phenotype. We have sequenced the entire CMD1 gene including 5Kb 5' to the transcriptional start site and have identified the minimal region in the gene necessary for muscle-specific transcription in primary cultures of embryonic chick skeletal muscle. The CMD1 promoter is silent in primary chick fibroblast cultures and in muscle cell cultures treated with the thymidine analog, BrdU. However, CMD1 and chicken myogenin, as well as chicken myf5 5, and MRF4 to a lesser degree, expressed in trans can activate transcription from the minimal CMD1 promoter in these primary fibroblast cultures. The promoter contains numerous E-box binding sites for CMD1 and the other myogenic factors, as well as a MEF-2 binding site. Surprisingly, muscle-specific expression, auto regulation and cross activation do not depend upon the presence of these E-box or MEF-2 binding sites in the CMD1 promoter. These results demonstrate that auto regulation and cross activation of the chicken MyoD promoter through the putative direct binding of the myogenic bHLH regulatory factor are, in fact, mediated through an indirect pathway that involves unidentified regulatory elements and/or ancillary factors.

2. The role of phosphorylation in the regulation of the avian MyoD, CMD1.

MyoD is a member of the basic helix-loop-helix (bHLH) family of muscle gene regulatory proteins that includes myogenin, myf5 and MRF4. These proteins have been shown to heterodimerize with E2A bHLH proteins, E12/E47, and to bind to a consensus sequence known as an E-box, CANNTG, the target for transcriptional activation by these myogenic regulators. MyoD is also a phosphorylated nuclear protein that is present in muscle cells prior to the transcriptional activation of the muscle-specific genes, many of which contain E-box elements in their regulatory regions. Phosphorylated chicken MyoD, called CMD1, produced in sf9 cells using the baculovirus system, is qualitatively similar to CMD1 isolated by immunoaffinity purification from primary cultures of embryonic chick breast muscle. Functional analysis of phosphorylated and dephosphorylated CMD1 produced in sf9 cells indicates that DNA binding of phosphorylated CMD1 is inhibited whereas binding in association with E12 is not affected. However, CMD1 binding alone is equally efficient when either dephosphorylated or bacterially expressed CMD1 is used in the assay. These results suggest that cellular phosphorylation changes the CMD1 homodimer-heterodimer equilibrium which, in turn, modulates and/or eliminates binding site competition between CMD1 homodimers and CMD1/E-protein heterodimers in the cell. Preliminary results indicate that the Drosophila MyoD homodimer is also regulated similarly. CMD1 is phosphorylated only on serine residues. We have narrowed the identification of the regulatory phosphoserine(s) to a cluster of 3 serines in the carboxy terminal region of CMD1.

3. Collaborative studies on the role of avian MyoD (CMD1) in myotome formation.

In mammalian development myf5 is expressed as the first myogenic factor in the dorsal medial region of the developing somites. MyoD is expressed one day later. However, in avian species, MyoD is expressed first followed by myf5. Formation of paraxial muscles in vertebrate embryos depends upon interactions between early somites and the neural tube and/or notochord. Removal of both axial structures results in complete loss of myotomal muscle, whereas hypaxial and limb muscle develop normally. Using our avian clones for MyoD (CMD1) and myogenin (cmgn), in collaboration with Dr. Bodo Christ (Institute of Anatomy, University of Freiburg) and Dr. Hans Arnold (Department of Cell and Molecular Biology, Technical University of Braunschweig), we have been able to show that chicken embryos with the neural tube surgically removed at the level of unsegmented paraxial mesoderm start to develop myotomal cells which express transcripts for the myogenic factors MyoD and myogenin. These cells also make desmin indicating that the initial steps of axial skeletal muscle formation can occur in the absence of the neural tube. However, a few days following the surgery, most myogenic cells gradually disappear and are almost undetectable at four days. We conclude that the neural tube is not required for the generation of the skeletal muscle cell lineage but may support the survival of the myotomal cell compartment.

4. The role of the E2A gene in myogenesis.

The myogenic factors bind DNA predominantly as heterodimers with E2A-related proteins based upon studies carried out in vitro with proteins made in E. coli or cell-free systems. The actual in vivo complex has been suggested from transfection studies in cell lines but the actual in vivo complex and the role of the E2A proteins in myogenesis has not been rigorously established. We have isolated the cDNA clones for the avian E2A gene products E12 and E47 and developed reagents to study the role of these E-proteins during myogenesis in primary chick muscle cultures. The E2A proteins are highly conserved between chicken and human and are closely related to the other E- proteins E2-2 (ITF2), E2-5(ITF1) and HEB. The Avian E2A proteins are expressed at much higher levels in muscle compared to liver or brain, as much as ten-fold higher. Immunofluorescent studies demonstrate that nuclear concentrations of E2A protein increase dramatically with muscle differentiation and this increase is also reflected an the mRNA level. Immunoprecipitation studies with whole cell or nuclear extracts from differentiated muscle indicate that E2A proteins are tightly complexed with MyoD and myogenin, consistent with the idea that these proteins function mainly as heterodimers.

To look more closely at the role of the E2A proteins in myogenesis an RNA antisense protocol was established. Previous work with ES cells missing the E2A gene (knockout) demonstrated that in this cell background the E2A gene products were not essential for muscle formation. This result was attributed to the redundant nature of the E-protein family. In the primary muscle cell lineage, however, the E2A gene products appear to be required for normal myogenesis, suggesting that the redundancy seen in ES cells is not present in primary chick myoblast cultures. Cotransfections with a five-fold molar excess of the antisense expression construct completely blocked activation of a CAT reporter construct with E2-5 binding sites. The same antisense constructs cotransfected with a beta-gal expression plasmid to mark transfected cells completely blocked myogenesis and this block could be reversed with an excess of the sense expression construct. The non-E2A E-proteins, therefore, cannot rescue this inhibition and this result strongly suggests E12/E47 play a role in the function of the myogenic factors. Mutants in the Drosophila E-like protein, daughterless, also show a defective muscle phenotype.

Studies on Drosophila Myogenesis.

The process of myogenesis in Drosophila is similar to that in vertebrates in that mesodermally established myoblasts withdraw from the cell cycle in G-0 and fuse to form the multinucleated muscle fiber. We have isolated the Drosophila MyoD homolog called Dmyd or nau. There is tremendous conservation at both the nucleotide and amino acid level in the basic helix-loop-helix region between the vertebrate myogenic factors and Dmyd. Unlike the vertebrates, however, there is only a single gene in the fly. The extremely conserved bHLH domain, with only six amino acid differences, suggested Dmyd would also convert mouse 10T1/2 cells to muscle, the standard assay for myogenic factor function. Surprisingly, conversion was roughly 100-fold less efficient than for the vertebrate factors. Dmyd would not dimerize efficiently with E12 or E47 although the homodimer would bind to a standard vertebrate E-box. Expression studies revealed Dmyd was a nuclear antigen expressed in a subset of muscle cells, at least one of which was incorporated into practically every muscle in the fly. However, the antigen and mRNA were transiently expressed and essentially vanished when muscle fibers were forming. In vertebrates one of the factors is continuously expressed in all skeletal muscle. In order to study the role of Dmyd in Drosophila myogenesis, we chose to ablate cells expressing Dmyd. This was accomplished using a cold-sensitive Ricin A-chain toxin expressed in a P-element construct under the control of a 8 Kb Dmyd promoter fragment. We had established that this promoter would direct beta-galactosidase expression in a pattern similar to the Dmyd protein. When the toxin is active there is a severe muscle disruption and this is under analysis. The Dmyd promoter driven beta-galactosidase expression suggests that certain muscle groups are more affected than others in the larva so this is being studied in crosses of the toxin ts lines with the Dmyd promoter driven beta-galactosidase lines.

We have identified the product of the daughterless gene, an E-like protein in Drosophila, as an in vitro partner for Dmyd. The daughterless homodimer and the heterodimer with Dmyd will bind a vertebrate E-box in gel shift assays. The most striking finding that supports the idea that daughterless and Dmyd form a heterodimer that plays a role in Drosophila myogenesis comes from cotransfection studies in vertebrate 10T1/2 cells. Even though expression of Dmyd or daughterless alone in 10T1/2 cells does not result in myogenic conversion, cotransfection of these factors results in conversion with an efficiency similar to the vertebrate myogenic factors alone. We now know that the effectiveness of Dmyd depends upon the proper partner protein.

Dmyd is a natural mutant which does not convert 10T1/2 cells to muscle in the absence of the proper partner protein. We have developed an entirely new assay to study dimerization specificity that has enabled us to look at the differences between Dmyd and vertebrate MyoD (CMD1) in their ability to dimerize with the vertebrate E-protein E12. Using a non-DNA-binding MyoD mutant with a normal HLH domain as a dimerization competitor in gel mobility shift assays in conjunction with various MyoD HLH mutants, nonhydrophobic amino acids were identified in the HLH domain that contribute to dimerization specificity with E12. The assay detected subtle differences in dimerization activity among the mutant MyoD proteins that correlated with their ability to activate transcription in vivo, but this correlation was not apparent in the absence of competitor. The identification of such nonhydrophobic residues enabled us to predict the differences in dimerization affinity among the four vertebrate myogenic factors with E12. The experiments confirmed the prediction. Furthermore, a high-affinity homodimerizing analog of MyoD was designed by a single substitution at one of these residue positions. These experimental results were strengthened when they were analyzed in terms of the crystal structure for the Max bHLHZip domain homodimer. This analysis has allowed us to identify those residues that form charged residue pairs between the two HLH domains of MyoD and E12 and determine the dimerization specificity of the bHLH proteins. In collaboration with Dr. Andy

Fire at the Carnegie Institution of Washington, we were able to predict and demonstrate that the Drosophila Dmyd, based upon the HLH charge interactions, had a good chance of rescuing a C. elegans MyoD lethal point mutation. Worms given the Drosophila gene appear normal.

Dr. Deborah Hursh has been involved in a separate project, as follows. The *Drosophila decapentaplegic (dpp)* gene encodes a TGF-β family member that participates in a signal transduction pathway leading to embryonic midgut morphogenesis. Her previous work showed that this pathway requires the action of the homeotic transcription factor *Ubx* and the product of the *dpp* gene itself in an indirect autoregulatory feedback loop. In order to better understand this autoregulation, she has dissected the *Ubx* -responsive promoter region of *dpp*. An 812 bp region in the 5' end of the gene directs spatially correct expression of a reporter construct. A "double switch" experiment was performed: all predicted *Ubx* binding sites are mutated to respond to a *bicoid*-type homeodomain recognition helix, and A *Ubx* c-DNA was altered to contain such a recognition helix (glutamine 50 to lysine 50) and introduced into the germ line of flies. The ability of this altered *Ubx* c-DNA to activate these mutated sites in vivo demonstrates that the action of *Ubx* on *dpp* represents a direct interaction of *Ubx* protein on *dpp* cis-regulatory DNA. It was found, however, that alteration of a particular homeodomain recognition site within the 812 bp reporter extinguished expression in a non-*Ubx* dependent manner. This particular binding site, while conforming poorly to the *Ubx* recognition site consensus, is a good site for the homeodomain transcription factor *pbx-1*, whose homolog in flies is the product of the *extradentical* (*exd*) gene. It is therefore possible that *exd* is a necessary co-factor for *Ubx* activation of *dpp*. Purified *exd* protein specifically protects the site predicted by consensus sequence in an in vitro footprint. Constructs that will specifically alter potential *exd* binding sites in the *dpp* promoter are planned, as well as a "double -switch" of the *exd* recognition helix to a *bicoid* recognition helix as described above for *Ubx*. Such experiments will establish that *exd* is also a direct activator of *dpp*. The possible role of *exd* as a target by which *dpp*'s autoregulation is carried out is also being investigated.

Publications:

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

PROJECT NUMBER
Z01 CB 05263-13 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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G. Mizuguchi	Visiting Fellow	LB	NCI
M. Fritsch	IRTA Fellow	LB	NCI
N. Hosakawa	Visiting Fellow	LB	NCI
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COOPERATING UNITS (if any)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8.25	7.75	0.5

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☐ (a1) Minors
☐ (a2) Interviews

B

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

Carl Wu's group has continued their studies on the regulation of heat shock gene expression, with emphasis on the structure and function of the heat shock transcription factor (HSF) and on the organization of the heat shock gene promoter in chromatin. The solution structure of the DNA binding domain of HSF, a helix-turn-helix motif, was determined by NMR spectroscopy, a first step in understanding the physical basis of sequence-specific recognition by HSF. Using the analytical ultracentrifuge, this group rigorously determined the affinity and stoichiometry of the interaction of the DNA binding domain of HSF with its cognate site and the thermodynamic parameters that govern site-specific binding. The regions of HSF required for the monomer-trimer transition of HSF were further mapped by exchanging domains between Drosophila HSF and human HSF, and the role of heat shock proteins in regulating the activity of HSF was clarified. Initial studies of post-translational modifications of HSF using mass spectrometry provided encouraging data on the feasibility of this direction of investigation. This group discovered an ATP-dependent activity that was necessary for the transcription factor-mediated disruption of nucleosomes on the chromatin fiber. Another regulatory function for chromatin structure was discovered in the abrogation of the heat shock response during the mitotic phase of the cell division cycle.

Project Description

Objectives: Regulation of heat shock gene expression

Regions of HSF regulating the monomer-trimer transition.

The DNA binding activity of Drosophila HSF (dHSF) and human HSF1 (hHSF1) is suppressed under normal conditions by a stabilization of the monomeric state of the transcription factor. Previously we had identified a C-terminal region including a leucine zipper motif on both HSF proteins whose integrity is required for monomer stability. In order to identify additional domains of HSF regulating monomer structure, we transiently expressed a number of dHSF deletion mutants in *Drosophila* SL2 cells by transfection, and tested their DNA binding activities and oligomeric properties. We also found that the temperature response of hHSF1 expressed in *Drosophila* cells was reset to the response of the host cell, while dHSF expressed in human 293 cells was constitutively active and trimeric, even when 293 cells were incubated at room temperature. We have exploited this phenomenon to investigate which domains of human HSF1 are necessary to confer proper regulation on dHSF in human 293 cells, by analysis of chimeric HSF proteins containing segments of hHSF1 substituting for corresponding parts of dHSF. We found that the N-terminal half of hHSF1 (containing the DNA binding and trimerization domains) fused to the C-terminal half of dHSF showed proper regulation of DNA binding activity in 293 cells, while the N-terminal half of dHSF fused to the C-terminal half of hHSF1 was constitutively active. These results suggest that the mechanism controlling formation of the latent HSF monomer operates on both the C-terminal and N-terminal regions of HSF. Detailed swapping analysis and mutagenesis are currently underway to precisely identify the amino acid residues necessary for suppression of HSF trimerization in human cells. This should allow a better understanding of HSF monomer formation, and suggest targets on HSF for the action of cellular regulatory factors.

Role of hsp70 in regulating HSF activity.

The intracellular level of free heat shock proteins, in particular the hsp70 protein family, has been suggested to be part of an autoregulatory loop by which the cell measures the level of thermal stress. According to this model, the DNA binding and oligomeric state of the heat shock transcription factor (HSF) is directly sensitive to the concentration of hsp70 protein. To test this model, we investigated the association between HSF and hsp70 by means of a co-immunoprecipitation assay. We found that hsp70 associates to a similar extent with both latent and active forms of HSF, but that this association was not significantly disrupted in the presence of ATP. Gel mobility shifts assays indicated that active HSF trimers purified from a bacterial expression system or present in a crude nuclear extract could not be significantly deactivated *in vitro* with purified hsp70 and ATP. In addition, elevated concentrations of heat shock proteins could not significantly inhibit induction of the DNA binding activity of endogenous HSF in cultured rat cells or in *Drosophila* SL2 cells. However, we found that deactivation of HSF to the non-DNA binding state could be accelerated by increased levels of heat shock proteins. Hence, while increased levels of heat shock proteins may modulate the kinetics of the oligomeric transition of HSF, the changing level of hsp70 and the other heat shock proteins is apparently insufficient to control the equilibrium between HSF monomers and trimers. Instead, we speculate that hsp70 may affect the transactivation function of HSF from the higher eukaryotes, as has been previous shown for the constitutively trimeric yeast HSF. This hypothesis will be tested in the incoming year.

Thermodynamic analysis of the interaction of the HSF DNA binding domain and its cognate site.

The high affinity binding of HSF is dependent on the formation of an HSF homotrimer, which interacts specifically with the heat shock response element (HSE), comprised of three inverted repeats of the 5 base-pair sequence _NGAAN_. In order to investigate the thermodynamic basis of the interaction

between HSF and HSE, we have overexpressed and purified a polypeptide [dHSF(33-163)] encompassing only the DNA-binding domain of HSF from *Drosophila*, and analyzed its binding to DNA by equilibrium analytical ultracentrifugation using a multi-wavelength scan technique. This analysis was conducted in collaboration with Dr. Marc Lewis (BEIP). We demonstrated that dHSF(33-163) could bind as a monomer with 1:1 stoichiometry to a synthetic 13 base–pair DNA containing a single _NGAAN_ sequence. The values of the thermodynamic parameters obtained from the temperature dependence of the equilibrium binding constants indicated that the changes of free energy for the binding of dHSF(33-163) to the wild-type site and a mutant DNA site were predominantly characterized by substantial negative changes of enthalpy. Binding to the wild-type DNA was characterized by a significant positive change of entropy while binding to the mutant DNA was distinguished by a negative change of entropy of comparable magnitude. The binding to the mutant DNA was also highly sensitive to increasing salt concentrations, indicating a dominance of ionic interactions. The sequence-specific, 1:1 binding of dHSF(33-163) to the _NGAAN_ sequence provides a basis for the future analysis of higher order interactions between HSF trimers and the HSE.

Solution structure of the DNA Binding Domain of Drosophila HSF.

The structure of the DNA binding domain of Drosophila HSF was determined by multi-dimensional multinuclear NMR spectroscopy, in collaboration with Dr. Ad Bax (Laboratory of Chemical Physics, NIDDK). The solution structure of the DNA-binding domain resembles that of the helix-turn-helix class of DNA binding proteins. The domain comprises a four-stranded antiparallel β-sheet, packed against a three-helix bundle. The second helix is significantly distorted and is separated from the third helix by an extended turn which is subject to conformational averaging on an intermediate time scale. Helix 3 forms a classical, amphipathic helix of 11 residues with polar and charged residues exposed to the solvent. Upon titration of the polypeptide with a DNA duplex containing a single nGAAn recognition site, resonance shifts in the backbone and Asn and Gln side chain amides indicate that helix 3 makes the most extensive contacts with DNA. We therefore propose that the third helix of the DNA-binding domain acts as the recognition helix of the heat shock transcription factor. Although study of the structure of the dHSF-DBD complex with DNA by either NMR or crystallography is hampered by the relatively low affinity of the monomeric binding domain, this work provides a first step towards an understanding of the structure of the protein-DNA complex, which, in turn may address the intriguing question of how three monomer binding domains of the HSF trimer are naturally aligned on the 15 bp heat shock element.

Post-translational modifications of HSF.

The laser desorption time-of-flight mass spectrometer installed in the Laboratory of Biochemistry has proved a valuable resource not only for the analysis of post-translational modifications of HSF but also for checking the precise mass of bacterially expressed protein domains. In several instances inconsistencies between the measured mass of the polypeptide and the molecular weight calculated from the predicted open reading frame revealed hidden errors in the DNA sequence of the recombinant clones. As a pilot experiment, we have compared the mass of HSF purified from E. coli and a Baculovirus expression system, and found substantial mass differences between the two proteins. This result suggests multiple post-translational modifications have occurred on the protein when it is expressed in insect cell system. We are currently using HPLC chromatography to isolate peptides from the bacterially expressed HSF after cleavage with hydroxylamine, and have identified the individual peptides on the basis of their masses predicted from the HSF open reading frame. This data will serve as a basis for a comparison of the corresponding peptides isolated from Drosophila HSF purified from non-heat shocked and heat shocked Drosophila cells. For this comparison, we are developing a

protocol to purify the naturally expressed Drosophila HSF using immunoaffinity chromatography as the key purification step. Further progress on the analysis of HSF modifications on the naturally expressed protein is anticipated in the coming year.

Discovery of an ATP-dependent chromatin remodeling activity.

We have developed an *in vitro* assay for the establishment of a nucleosome-free region in chromatin, using a chromatin assembly extract prepared from *Drosophila* embryos, plasmid DNA and purified transcription factors. As a model system, we analyzed the *Drosophila* hsp70 promoter, which contains sites for interaction with HSF, the heat shock transcription factor, GAGA factor, a constitutively expressed transcription factor that binds to GA/CT-rich sites present in many *Drosophila* genes, TFIID, the TATA binding general transcription factor complex, and an RNA polymerase II molecule paused after synthesizing a short transcript. GAGA factor, TFIID, and RNA polymerase II are active and can associate with heat shock promoters *in vitro* in the absence of a heat shock stimulus. Thus, these proteins are potential candidates for the establishment of an accessible promoter complex poised to respond to the binding of the activated, trimeric form of HSF. We have determined the role of the GAGA factor in altering chromatin structure. The introduction of GAGA protein during or after nucleosome assembly *in vitro* was found to result in a disruption of nucleosome structure at the hsp70 promoter. The disruption is characterized by hypersensitivity to DNase I digestion and a realignment of adjacent nucleosomes. Importantly, the disruption of chromatin is facilitated by ATP hydrolysis, suggesting the involvement of an energy-dependent pathway for chromatin remodeling. We are currently involved in the purification of this ATP-dependent activity from Drosophila embryo extracts.

Cell cycle regulation of the heat shock response

Although the periodic inactivity of genes at the mitotic stage of the eukaryotic cell division cycle is an established concept in the biological literature, surprisingly little is known of the mechanisms that govern this cyclic form of genetic repression. We have investigated the heat shock response of Hela cells blocked in mitosis with the microtubule-targeting drugs nocodozole and taxol, and found that the cells are unable to induce the synthesis of heat shock RNAs specifically in this phase of the cell cycle. Furthermore, cell viability appears to be particularly decreased when a heat stress in applied in mitosis, but not at interphase. We are currently investigating the molecular basis of the mitotic repression of heat shock gene transcription, and the possibility that this heat shock-sensitive period in the cell cycle may offer a therapeutic window for combining heat treatments with anti-mitotic drugs such as taxol.

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Lis J, Wu C. Protein traffic on the heat shock promoter: parking, stalling, and trucking along, Cell 1993;74:1-4 .

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Wu C, Clos J, Giorgi G, Haroun RI, Kim S-J, Rabindran SK, Westwood JT, Wisniewski J, Yim G. Structure and regulation of heat shock transcription factor. In: Morimoto RI, Tissieres A, Georgopoulos C. eds. The biology of heat shock proteins and molecular chaperones. New York: Cold Spring Harbor Laboratory, 1994;395-416.

Vuister G, Kim S-J, Wu C, Bax A. NMR evidence for similarities between the DNA binding regions of Drosophila melanogaster heat shock factor and the helix-turn-helix and HNF-3/forkhead families of transcription factors, Biochemistry 1994;33;10-6.

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Kim SJ, Tsukiyama T, Lewis M, Wu C. The Interaction of the DNA-binding domain of Drosophila heat shock factor with its cognate DNA site: a thermodynamic analysis using analytical ultracentrifugation, Protein Science, in press.

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

PROJECT NUMBER
Z01 CB 05264-13 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Biosynthesis Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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.)

Activation of particular IAP elements is likely to be determined by the methylation state of their LTRs as well as the availability of appropriate transcription factors. Increased expression of IAP elements in plasmactyoma cells compared with LPS-stimulated normal B-cells is accompanied by extensive hypomethylation of IAP sequences. Subsets of these sequences can be detected with oligonucleotide probes based on sequences in expressed IAP elements. We have used these probes to examine the methylation state of the endogenous IAP proviral sequences and have found that multiple common IAP loci were hypomethylated in four independently established plasmacytomas. Many of the same IAP loci were hypomethylated in primary plasmacytomas induced by two different methods. However, not all IAP elements were demethylated even when their LTR sequences were very similar, suggesting that the methylation state of the proviral DNA is determined by their position in the genome. In primary tumors, hypomethylation occurred more frequently in DNA flanking the IAP elements than it did in established tumors, suggesting that for some loci hypomethylation may be extended into the IAP LTRs during progression of the tumors. Multiple common IAP loci were also found to be hypomethylated in other types of B-cell tumors. In these tumors the IAP hypomethylation patterns reflected the developmental state of the B-cell from which the tumors were derived.

Project Description

Objective:

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

Major Findings:

Mouse plasmacytomas express higher levels of RNA transcripts from endogenous intracisternal A-particle (IAP) proviral elements than do lipopolysaccharide-stimulated normal lymphocytes. Lymphocytes express a limited and highly characteristic set of IAP elements (lymphocyte-specific [LS] elements). Expression of IAP elements in transformed B cells is selective for a different set of regulatory sequence variants (plasmacytoma [PC] elements) than those expressed in normal B cells, although LS elements continue to be expressed in many plasmacytomas. Activation of particular IAP elements is likely to be determined by the methylation state of their LTRs as well as the availability of appropriate transcription factors.

We have used probes representing R-region variants of expressed IAP elements and the presence of a conserved methylation sensitive *Hae*II site in the 5'LTR to determine the methylation status of individual IAP proviruses. Oligonucleotide probes representing LS- and PC-specific sequence variants detected multiple common hypomethylated IAP proviral loci in four independently derived established plasmacytomas (MOPC315, MOPC104E, MOPC21, and MPC11). However, not all IAP elements were hypomethylated even when their LTR sequences were very similar, leading to the conclusion that the methylation state of many IAP elements is determined by their position in the genome. Most of the IAP proviral sequences were hypomethylated at sites in the LTR or close to it in the flanking DNA. For the few loci for which hypomethylation occurred in the DNA flanking the IAP element, it appeared to occurred at a common site. The results show that IAP element hypomethylation in established plasmacytomas does not occur entirely randomly.

We have also used the IAP subset specific probes to examine whether distinctive patterns of hypomethylation are also present in primary plasmacytomas. We examined 7 primary plasmacytomas, 3 of which were induced with pristane, and 4 of which were induced with a viral vector carrying c-*myc* and v-Ha-*ras* genes. The primary tumors were isolated as diffuse nodular implants from mesenteric surfaces. Many of the same IAP loci that were hypomethylated in established plasmacytomas were also hypomethylated in primary tumors. In primary tumors hypomethylation appeared to occur more frequently in the flanking DNA rather than in the IAP elements themselves, suggesting that for some loci hypomethylation may be extended into the IAP LTRs during progression of the tumors.

We have found that hypomethylation of multiple common IAP loci also occurs in B-cell tumors other than plasmacytomas. The patterns appeared to be related to the B-cell developmental stage at which the tumors arose. Thus, B-cell lymphomas derived from mature B-cells had a pattern of IAP gene hypomethylation similar to that in plasmacytomas, while lymphomas derived from early B-cells had a pattern more similar to normal stimulated B-cells. However, hypomethylated loci unique to tumors of different types were also found. Thus, the hypomethylated IAP elements may serve as reporters of genomic hypomethylation during B-cell development and transformation that reflect regions of cellular gene activity that are not detected by other means.

Publications:

Lueders KK, Fewell JW, Morozov VE, Kuff EL. Selective expression of intracisternal A-particle genes in established mouse plasmacytomas, Mol Cell Biol 1993;13:7439-46.

Lueders KK, Fewell JW. Hybridization of DNA in dried gels provides increased sensitivity compared with hybridization to blots, BioTechniques 1994;16:66-7.

Lueders KK, Kuff EL. Intracisternal A-particle (IAP) genes show similar patterns of hypomethylation in established and primary mouse plasmacytomas, Current Topics in Microbiology and Immunology, in press.

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

PROJECT NUMBER
Z01 CB 05265-12 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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 STAFF YEARS:	PROFESSIONAL:	OTHER:
3	3	0

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☐ (a) Human ☐ (b) Human tissues x☐ (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.

Secretion of norepinephrine by permeabilized PC12 cells can be stimulated by the addition of either GTPγS or ATPγS. Stimulation of secretion by GTPγS results from its binding to and activating a low molecular weight GTP-binding protein. In contrast, stimulation by ATPγS appears to result from its conversion to GTPγS by nucleoside diphosphate kinase. The time course of ATPγS-stimulated secretion and the relatively poor inhibition of ATPγS-stimulated secretion by GTP suggest that nucleoside diphosphate kinase may interact directly with this GTP-binding protein.

14-3-3 proteins appear to a critical role in Ca^{2+}-stimulated norepinephrine secretion by permeabilized chromaffin cells, but their precise role in this process remains to be determined. 14-3-3 proteins have been reported to regulate proten kinase C. We have found that the reported effects of 14-3-3 proteins on protein kinase C activity result not from an interaction of 14-3-3 proteins with protein kinase C but rather with the histone used as a substrate in the protein kinase C assays. When other substrates are used, 14-3-3 proteins have no effect on protein kinase C activity. Thus, 14-3-3 proteins do not appear to regulate protein kinase C.

We have found that pertussis toxin-modification of unstimulated PC12 cells results in about a 50% inhibition of protein phosphatase 2A. This decrease in phosphatase activity does not result from a decrease in the level of protein phosphatase 2A but rather from a decrease in its specific activity. This decrease in activity appears to result from a chemical modification of the phosphatase. Experiments are being conducted to determine the nature of this modification.

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 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 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 K^+-depolarization results in the 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 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 Ca^{2+} and nucleotide concentrations and to introduce proteins into the cell. We use these permeabilized cells to investigate the roles of Ca^{2+}, GTP-binding proteins, the cytoskeleton, and protein phosphorylation in secretion.

Major findings:

14-3-3 proteins: Catecholamine secretion by digitonin-permeabilized chromaffin cells is Ca^{2+}-dependent. Incubation of these permeabilized cells in the absence of Ca^{2+} results in a progressive loss of proteins and secretory activity. The addition of cytosolic proteins prevents this loss of Ca^{2+}-dependent secretion. We have previously shown that 14-3-3 proteins play a key role in this restoration. We have performed several types of experiments to try to understand the role of 14-3-3 proteins in the secretory process. It has been reported that a Ca^{2+}-sensitive phospholipase A_2 is a form of 14-3-3 proteins. However, we found that 14-3-3 proteins, isolated on the basis of their ability to stimulate secretion, have no significant phospholipase A_2 activity. 14-3-3 proteins have also been reported to be both stimulators and inhibitors of protein kinase C. We have found that 14-3-3 proteins stimulated histone phosphorylation by protein kinase C, but they had no effect on protein kinase C phosphorylation of either myosin light chains or a synthetic peptide substrate specific for protein kinase C. Similarly, we found that 14-3-3 proteins inhibited the dephosphorylation of ^{32}P-histone by a cell extract but not the dephosphorylation of ^{32}P-myosin light chains. Cross-linking experiments and affinity chromatography demonstrated that 14-3-3 proteins bind to histones but not to myosin light chains or to protein kinase C. Thus, the reported effects of 14-3-3 proteins on protein kinase C activity appear to result from 14-3-3 proteins binding to histone, and contrary to what has been suggested by other investigators, 14-3-3 proteins do not appear to regulate protein kinase C activity.

Stimulation of secretion by GTPγS: Secretion of norepinephrine by digitonin-permeabilized PC12 cells can be stimulated by the addition of Ca^{2+} or GTPγS but not GTP. While secretion in the presence of saturating Ca^{2+} is not affected by GTPγS, secretion in the absence of Ca^{2+} is stimulated 2 to 3 fold by the addition of GTPγS. This stimulation by GTPγS does not appear to result from Ca^{2+} release, activation of protein kinase C, or stimulation of phospholipase A_2. 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 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. Our data indicate that G_e in PC12 cells is a low molecular weight GTP-binding protein rather than a heterotrimeric G-protein.

The addition of ATPγS to digitonin-permeabilized PC12 cells also stimulates Ca^{2+}-independent norepinephrine secretion. We have previously reported preliminary data that suggested that the effect of ATPγS on secretion might result from its conversion to GTPγS by nucleoside diphosphate kinase. We have now obtained more direct evidence that this conversion occurs. Incubation of permeabilized PC12 cells in a sucrose containing media resulted in a preferential loss of ATPγS-stimulated norepinephrine secretion and a decrease in the level of nucleoside diphosphate kinase. The addition of purified nucleoside diphosphate kinase to the depleted cells partially restored ATPγS-stimulated secretion. The time course of ATPγS-stimulated secretion and the relatively poor inhibition of ATPγS-stimulated secretion by GTP suggest that either the formation of GTPγS from ATPγS by nucleoside diphosphate kinase occurs in close proximity to the GTP-binding protein responsible for stimulating secretion or that nucleoside diphosphate kinase interacts directly with this GTP-binding protein.

Inhibition of protein phosphatase 2A by pertussis toxin: A number of cell surface receptors are linked to their intracellular effector systems through heterotrimeric G-proteins. Pertussis toxin ADP-ribosylates the α-subunits of the G_i and 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.

Pertussis toxin modification of P12 cells results in both a decrease in cytoskeletal F-actin and an enhancement of secretory activity. As reported previously, 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 can account for both the decrease in cytoskeletal F-actin and the increase in secretory activity. The pertussis toxin-induced decrease in protein phosphatase 2A activity does not result from a decrease in the level of protein phosphatase 2A but rather from a decrease in its specific activity. Protein phosphatase 2A from pertussis toxin-modified PC12 cells binds more tightly to an ion exchange column than does protein phosphatase 2A from control cells. This suggests that one of the three subunits of this phosphatase has been modified. Experiments are being conducted to determine the nature of this modification.

Publications:

Rhoads AR, Pauri R, Vu ND, Cadogan R, Wagner PD. ATP-induced secretion in PC12 cells and photoaffinity labeling of receptors. J Neurochem 1993; 61:1657-66.

Vu ND, Wagner PD. Stimulation of secretion in permeabilized PC12 cells by adenosine 5'-[γ-thio]triphosphate: possible involvement of nucleoside diphosphate kinase. Biochem J 1993;296:169-74.

Chen F, Wagner PD. 14-3-3 proteins bind to histone and affect histone phosphorylation and dephosphorylation FEBS Lett (in press).

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

PROJECT NUMBER
Z01 CB 05267-10 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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G. Mukhopadhyay	Visiting Fellow	LB	NCI
M. Lobocka	Visiting Fellow	LB	NCI
H. Lehnherr	Visiting Fellow	LB	NCI
J. Dibbens	Visiting Fellow	LB	NCI
V. Rybchin	Volunteer	LB	NCI

COOPERATING UNITS (if any)
Drs. J. Kaguni & K. Carr, Michigan State University, MI; K. Rudd, National Library of Medicine, MD; V. Rybchin & A. Svarchevsky, State Technical University, St. Petersburg, Russia.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Microbial Genetics and Biochemistry Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.4	6.4	

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☐ (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.)

Two prophages, P1 and N15, belong to the important class of plasmids whose members can be stably maintained in E. coli despite low copy numbers. Since the previous annual report we have proceeded in new directions in our studies of P1 plasmid maintenance and have initiated a collaborative study of N15. N15 is the unique example of a plasmid residing in E. coli whose structure, like that of eukaryotic chromosomes, is linear. Several of our biophysical studies of P1 replication initiation and iteron-mediated control have been published recently. Work in progress is providing a domain analysis and a characterization of protein-protein interactions involving the multifunctional P1 replication initiator, RepA. The range of plausible models for active partition and forstringent replication control has been narrowed by our recently published evidence for the partitionability of non-replicating DNA elements. We have shown that a frequently encountered dysfunction of the P1 partition mechanism can be turned into a powerful tool for selecting partition-defectives. Certain of these mutants are currently being examined in detail. Of two P1-encoded proteins that we have determined can addict E. coli to the presence of P1, one has been purified and for the other we have developed an in vitro assay. Dependency of addiction on the bacterial protease ClpXP is the subject of a recently submitted article. In the course of a program initiated this year to compile, correct, extend, and analyze all available P1 DNA sequence data, new plasmid and bacterial genes have been identified, certain of which will be studied further. We also note here the sequencing and preliminary characterization of regulatory genes involved in N15 lytic growth and mini-N15 plasmid maintenance.

Project Description

I. DNA Replication

Domains of RepA Association

RepA, the replication initiator protein of plasmid P1, exists in solution as a mixture of monomers and dimers, but it is only the monomeric form that binds DNA specifically. DNA-bound monomers can dimerize and thus can pair (synapse) RepA-DNA complexes. It is the pairing reaction which controls P1 DNA replication. The dimers can bind DNA only nonspecifically but are proficient in pairing. These results indicate that the association of free monomers (dimerization) interferes with the DNA binding domain so that only nonspecific binding can occur at high protein concentrations. Because of the crucial role of RepA association in DNA binding and in replication control, we initiated a project this year to study RepA association in vivo using an assay that does not depend upon RepA binding to DNA. The initial results indicate that both wild type RepA and mutant RepAs that are defective in DNA pairing can dimerize in vivo. These results suggest that the dimerization and pairing domains are functionally distinct. The assay has allowed us to isolate dimerization-defective mutants of RepA that should permit us to identify the dimerization domain. We intend to examine the mutant proteins for pairing activity with the aim of testing our hypothesis that the dimerization domain is physically distinct from that used in pairing. We also intend to use the dimerization assay to determine whether or not chaperones activate DNA binding of RepA by dimer dissociation. Finally, we also intend to determine whether the dimerization domain contributes to cooperative binding of RepA at its multiple sites in the origin (side-by-side cooperativity). Preliminary evidence for weak cooperative binding ($w \leq 2$) has been obtained using linear fragments with two iterons.

Role of DnaA in P1 Plasmid Replication

Last year we reported that DnaA, the initiator protein of E. coli chromosomal replication, can help P1 plasmid replication in two ways: by improving DNA binding activity of RepA and by opening the strands of P1 origin DNA. To understand whether RepA-DnaA cooperations involve protein-protein interactions or whether they are mediated through DNA, we are assaying RepA-DNA interactions both in the presence and absence of DNA using ELISA and magnetic bead technologies. The initial results indicate that the two proteins can interact specifically without requiring DNA. To establish the significance of this finding in vivo, we intend to isolate RepA mutants that would require the DnaA protein but not its specific DNA binding sites in the P1 origin for plasmid replication. A second goal of this project is to determine whether DnaA can unpair RepA-mediated DNA pairs and thus can have a positive regulatory role in P1 plasmid replication.

II. Partition

Dysfunction of the P1 partition system as a powerful tool for the study of par functions

In the previous annual report we noted a striking and unexpected dysfunction of the P1 partition system that depends upon the context of *parS* (the P1 plasmid centromere) and upon the concentration of the P1-encoded protein ParB, which binds tightly to parS. Constructs in which parS had been cloned in any of several positions in the vector pGB2ts could not be established in bacterial hosts that supply even levels of ParB much below those present in a P1 lysogen. Mutants altered in *parB* that were incapable of causing this acute plasmid destabilization could be readily isolated. We suggested, on the basis of the few such mutants then analyzed, that the affected regions of ParB are critical for binding to *parS*. This assessment underestimated the variety of mutants obtainable. We have since isolated binding-proficient, destabilization-negative mutants separately altered in five regions of the ParB coding region, including one contained ParB protein capable of dimerization; not all dimerization-proficient extracts could bind to

parS. These findings suggest that binding proficiency resides in the dimer. Certain of the mutant ParB footprints on *parS* appear similar, if not identical, to those of the wild type, but all the mutants analyzed failed to complement the partition defect in a miniP1 with a truncated *parB* gene. We are currently analyzing further the mutants that retain the capacity for binding to *parS in vitro,* as these are likely to be informative about features of ParB required for subsequent steps in partition.

III. Addiction

Purification of Phd

We previously identified two P1-encoded proteins that together are responsible for addicting bacteria to the presence of P1 plasmid. The product of *phd* prevents host death by the product of *doc* which would otherwise kill a P1 lysogen. Doc does inflict death on curing, i.e. when a host loses its resident P1. Much of the effort on this project recently has been directed towards purifying Phd.

A role for protease ClpXP in P1 addiction

Previous genetic experiments suggested that the specific ATP-dependent bacterial protease, ClpXP, is essential for expression of the addiction phenotype. In the period of this report we provided biochemical evidence that Phd is a specific substrate of this highly selective protease. Phd is now being used in other laboratories (including that of S. Gottesman, LMB, NCI) in studies of ClpXP specificity.

An assay for Doc

As Doc kills bacteria only from within, the purification of the protein must rely on some other phenotype than Doc's lethality. A DNA band shift assay for doc has been developed on the basis of our observation that Phd binding to the promoter region of the addiction operon requires Doc. Presumably both proteins participate in autogenous regulation.

IV. P1 Genome Organization

From 1977 on we have undertaken periodic updates of the genetic map of bacteriophage P1. This year (in a collaboration with Kenn Rudd of the NLM) we have embarked on the more ambitious project of compiling from various sources an annotated sequence file of contigs that now comprise 40% of the physical map. The contigs are joined by stretches of Ns interrupted at appropriate locations by recognition sequences of 10 restriction endonucleases. These have been corrected and extended to meld neighboring contigs and analyzed to identify sites and potentially functional open reading frames (orfs) not previously recognized.

The analysis, although still incomplete, has revealed that within a segment not much larger than 1% of the P1 genome are clustered four regions homologous to scattered bacterial sequences. One of these is an orf we name *hoe* for homolog of *holE*, the structural gene for the theta subunit of the DNA polymerase III, the major replicative enzyme of *E. coli*. The function of theta remains obscure. If *hoe* can be shown to affect bacterial or P1 DNA replication, it may aid in revealing the elusive role of theta.

V. Regulatory Genes of the Linear Plasmid Prophage of N15

A collaboration was initiated this year with the laboratory of Valentin Rybchin (St. Petersburg, Russia) with the aims of identifying functions critical for the maintenance of N15 as prophage and rescuing from obscurity the body of work on N15 that has appeared only in the Russian language or is unpublished. During a brief visit of Rybchin to this laboratory, we succeeded in demonstrating major features of N15

that reveal significant differences and similarities with respect to P1 and λ. Adjacent regulatory genes of N15 with homology to the *c*I repressor gene and Q anti-terminator genes of λ, respectively, were cloned and sequenced. Unexpectedly, the *c*I-like gene was shown to be separate from the site of an N15 repressor mutation selected here that confers thermoinducibility. The *c*I-like gene appears to control plasmid replication which, as in P1, is Dam methylase-dependent, although we were able to select an N15 mutant that overcomes this dependency. We found plasmid replication of N15 to be independent of the host DnaA protein, unlike plasmid replication of P1 and of other low-copy-number plasmids. These results suggest the extent to which familiar components may be organized or function in novel ways in this unique coliphage and provide some necessary ground-work for eventual studies of how a linear plasmid is partitioned.

Publications:

DasGupta S, Mukhopadhyay G, Papp PP, Lewis MS, Chattoraj DK. Activation of DNA binding by the monomeric form of the P1 replication initiator RepA by heat shock proteins DnaJ and DnaK, J Mol Biol 1992;232:23-34.

Papp PP, Chattoraj DK, Schneider TD. Information analysis of sequences that bind the replication initiator RepA, J Mol Biol 1993;233:219-30.

Mukhopadhyay G, Carr KM, Kaguni JM, Chattoraj DK. Open-complex formation by the host initiator, DnaA, at the origin of P1 plasmid replication, EMBO J 1993;12:4547-54.

Lehnherr H, Maguin E, Jafri S, Yarmolinsky MB. Plasmid addiction: genes *doc* and *phd* of bacteriophage P1 that respectively cause death on curing of prophage and prevent host death when prophage is retained, J Mol Biol 1994;233:414-28.

Treptow N, Rosenfeld R, Yarmolinsky M. Partition of nonreplicating DNA by the *par* system of bacteriophage P1, J Bacteriol 1994;176:1782-6.

Lobocka M, Hennig J, Wild J, Klopotowski T. Organization and expression of the *Escherichia coli* K-12 *dad* operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase, J Bacteriol 1994;176:1500-10.

Papp PP, Chattoraj DK. Missing-base and ethylation interference footprinting of P1 plasmid replication initiator, Nucleic Acids Res 1994;22:152-7.

Mukhopadhyay G, Sozhamannan S, Chattoraj DK. Relaxation of replication control in chaperone-independent initiator mutants of plasmid P1, EMBO J 1994;13: in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05268-07 LB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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,

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 STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	

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.)

We continued research on the molecular mechanism of meiotic recombination in the yeast Saccharomyces cerevisiae, with a focus on two aspects of this complex biological event. We have examined the initiation event in meiotic recombination, the formation of double-strand DNA breaks, with the aim of identifying the enzyme(s) responsible for break formation, their substrate requirements, and the factors that control the location and frequency of breaks. We also are studying the relationship between recombination and chromosome pairing, with the aim of identifying sites and factors important for the chromosome pairing and recombination events that occur during meiosis.

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 chromosomal location of double-strand breaks.

In yeast, meiotic recombination is initiated by the formation of DNA double-strand breaks (DSB) at a specific time during meiosis I prophase. We (Michael Lichten and Carol Wu) have shown that DSB occur exclusively at sites that display hypersensitivity (HS) to digestion of chromatin by the non-specific endonuclease DNase I. Conversely, all sites in chromatin that are DNase I-HS suffer DSB during meiosis. These findings imply that accessibility of DNA in chromatin plays an important role in determining where meiotic recombination initiates. However, since the majority of DSB/DNase I-HS sites occur in potential promoter regions, it is also possible that the act of transcription initiation or a sequence motif common to yeast promoters determines where DSB occurs, and that the association between DSB sites and open sites in chromatin is coincidental rather than causative.

We (Michael Lichten and Carol Wu) tested this suggestion by examining gene conversion, DSB, and chromatin structure at the *PHO5* locus. *PHO5* transcription is regulated by altering chromatin structure in its promoter. When the gene is repressed, positioned nucleosomes occlude the entire *PHO5* promoter region. Derepression is accomplished by the disruption of 3-4 of these nucleosomes, rendering a 5-600 base pair upstream region nuclease hypersensitive. This chromatin transition is transcription-independent, as it still occurs in strains where the *PHO5* TATA box is deleted and transcript levels are reduced 15 to 30-fold. We found that when nucleosomes occlude the region upstream of *PHO5*, DSB occur infrequently, and a genetic marker within *PHO5* displays a low frequency of gene conversion. When *PHO5* is induced and upstream nucleosomes disrupted, both upstream DSB and gene conversion increase markedly, and DSB occur throughout the region of nucleosome disruption. Identical frequencies and distributions of DSB are observed in strains with a wild-type *PHO5* promoter and in strains that harbor a deletion of the *PHO5* TATA box. We conclude that chromatin structure, rather than DNA sequence motifs or the transcriptional state of a gene, plays a primary role in determining where meiotic recombination events initiate.

B. Fine-structure mapping of DSB sites and determination of the nature of the break.

In preparation to a search for the activity responsible for DSB formation, Jianhua Liu has further characterized the nature of breaks that occur during meiosis. Most studies of meiosis-induced DSB have made use of the *rad50s* mutation, which blocks the 5'-3' recision of DSB ends and subsequent repair of breaks that occurs in wild-type cells. It has been suggested that, in wild-type cells, the primary recombinogenic lesion is not DSB, but rather single-strand nicks, and that only in the *rad50s* mutant background are these lesions processed to form DSB. Dr. Liu has used 2-dimensional agarose gel electrophoresis to demonstrate that the primary meiosis-induced lesion in wild-type cells is a double-strand DNA break, and that single-strand nicks do not occur at levels above the background seen in mitotic cells. Dr. Liu has also refined blotting and probing techniques to a level of sensitivity and

resolution that will allow mapping of DSB sites at the nucleotide level. Preliminary studies of two DSB hot-spots (the *ARG4* promoter region and the *YCL47c-YCL48w* intergenic region) indicate that breaks occur at multiple sites within a 150 bp region at both loci. Precise mapping of these break-points on both strands of the duplex will be completed in the near future. The results of these studies will indicate whether or not the DSB-forming enzyme(s) display target-sequence preference, and will also determine the strand overlap nature of the cleavage event. We plan to use this information in the design of model substrates for use in the isolation and characterization of meiosis-specific activities responsible for DSB formation.

C. The relationship between chromosome pairing and meiotic recombination.

Alastair Goldman has studied meiotic gene conversion between dispersed sets of homologous sequences (ectopic recombination), and has shown that ectopic recombination between sequences located on nonhomologous chromosomes (which do not pair during meiosis) occurs 5 to 20 times less frequently than does 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 paired homologs. He is currently making use of this observation to determine the chromosome elements that are responsible for homolog pairing. He is constructing specific translocations of chromosome III sequences to chromosome VIII, and examining, in a translocation heterozygote, the frequency of ectopic recombination between homologous sequences located on the non-translocation copies of chromosomes III and VIII. The presence of pairing elements in the translocated segment will increase the frequency of pairing, and thus the frequency of ectopic recombination, between these two heterologous chromosomes. These studies will allow us to determine whether or not specific chromosomal elements mediate homolog pairing. If so, this approach will be used to isolate the sequences that comprise these elements and to identify trans-acting factors necessary to their function.

Publications:

Wu TC, Lichten M. Meiosis-induced double-strand break sites determined by yeast chromatin structure, Science 1994;263:515-8.

Wu TC, Lichten M. Position effects in meiotic recombination. In: Haseltine FP, Heyner S, eds. Meiosis II: contemporary approaches to the study of meiosis. Washington DC: American Association for the Advancement of Science, 1993;19-36.

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;9:1111-20.

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

PROJECT NUMBER
Z01 CB 05271-03 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

C. Vinson	Senior Staff Fellow	LB	NCI
D. Krylov	Visiting Fellow	LB	NCI
M. Olive	Visiting Fellow	LB	NCI
C. Dezan	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any)
L. Bruggeman, P. Klotman, H. Armheiter, NIH; P. Johnson, Frederick Cancer Research Development Center; K. Yamada, NIH; J. Barchi, NCI

LAB/BRANCH
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SECTION
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	

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☐ (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.)

Eukaryotic gene expression is regulated by a complex assortment of different proteins associating with each other. This coming together of different proteins is structurally a subtle and largely unknown field. However, an understanding of the energetics of protein-protein interactions promises to be an important area for the design of novel molecules that disrupt gene regulation with possible future therapeutic consequences. We have been examining the structural and thermodynamic basic of dimerization of leucine zipper's, a coiled coil dimerization motif essential for the function of two classes of oncogenic DNA binding proteins, the bZIP and bHLH-Zip motifs.

During the last year my group has generated structural rules that govern the specificity of leucine zipper dimerization. Energetic parameters were obtained by measuring the change in ellipticity upon thermal melting of pure proteins. The main thrust of my group is to use these protein design rules to generate dominant-negative proteins. Initial experiments show that designed dominant-negative proteins preferentially heterodimerize with the cellular bZIP protein C/EBP and inactivate the function of C/EBP as assayed in a transient transfection assay. Additional versions of this basic strategy are being performed to determine which biochemical properties of these proteins are relevant biologically. We plan on expressing these dominant-negative proteins in transgenic mice to examine any possible phenotypes.

Project Description

Objectives

The design of dominant-negative proteins to explore the function of bZIP proteins in mammalian development and physiology.

Major Findings:

The bZIP Motif: Design of a Dominant-Negative.

A. Dominant-negative inhibits CEBP function by binding non-CEBP DNA

Last year we described a designed protein containing a mutated leucine zipper that preferentially heterodimerized with CEBP. To inhibit CEBP binding to a natural DNA target, we replaced the normal DNA binding region of our mutated protein with the DNA binding sequence from a plant bZIP protein. The designed protein was able to heterodimerize with CEBP and bind a non-CEBP DNA sequence. In transient transfection assays, this protein was able to inhibit the function of the three CEBP family members tested. This inhibition was dependent on the appropriate leucine zipper sequence as expected. In our hands, the naturally occurring protein CHOP 10, reported to be a dominant-negative of CEBP, is not functional.

B. Dominant-negative inhibits CEBP but does not bind DNA

A conceptual problem with the dominant-negative described in the previous section is that the heterodimer will bind an alternative sequence in the genome with unknown consequences. This concern prompted us to design a heterodimerizing dominant-negative (504) that did not need the additional stabilization provided by binding DNA. In an attempt to achieve this, we extended the leucine zipper region into the DNA binding region of the dominant-negative. This increases the dimerization constant of our dominant-negative a thousand fold. The 504/CEBP heterodimerize dissociates with a 10 pM dissociation constant, ten times higher than the 100 pM dissociation constant of CEBP homodimer bound to DNA. We are presently testing this protein in transient transfection assays and hope to use this construct in transgenic mice.

The bZIP Motif: Biochemistry

A. Leucine zipper stability and dimerization specificity

We have generated a large number of systematic mutant proteins that examine the importance of the **e** and **g** positions (the **g**↔**e'** interaction) of a coiled coil to dimerization stability. These mutant proteins have allowed the identification of a particular amino acid pair (E↔R) that are the most stabilizing and other pairs (E↔E) that are the most destabilizing. We are extending these studies to enrich our understanding of the structural determinants of the leucine zipper motif.

B. Determination of the physical nature of the coupling energy

Our biophysical experiments indicated a large coupling energy between the E↔R pair. We will examine the nature of this coupling energy by monitoring the effect of salt concentration and pH using both the CD and NMR machines. Using C13 labeling of the glutamic acid residues and determining NMR

spectra at different pH's and salt concentrations, we will be able to determine which fraction of the coupling energy is a consequence of electrostatic interactions between E and R. We have been able to generate C13 labeled proteins and, in collaboration with Joe Barchi, are determining the C13 NMR spectra of five different proteins. These fundamental studies will help in better understanding the energetic determinants driving leucine zipper formation.

C. Design of an inducible dimerization domain

Our extensive mutagenic study of leucine zippers did not include the phosphorylated amino acids, threonine, serine, and tyrosine. We expect that phosphorylation of these three amino acids will affect the stability of the leucine zipper. We are designing leucine zippers that contain a perfect PKA phosphorylation site with the serine in the g position. Our hope is that after phosphorylation, the structure will become more stable thus creating a phosphorylation dependent dimerization domain that could be of interest as an inducible system. Our initial proteins express well allowing biophysical studies but they are more stable than we had hoped. The melting temperatures are 50°C and 80°C. We are presently redesigning them to achieve melting temperatures around 30°C, thus allowing the kinase to phosphorylate an unhelical protein.

D. Design of a two-headed bZIP protein

The DNA binding domain of all bZIP proteins is N-terminal of the leucine zipper. We are exploring the design of a protein that additionally contains a C-terminal DNA binding domain. Campbell and colleagues have designed a bZIP protein with the sequence reversed. This potential double-headed protein could be designed with the ability to sequester two adjacent sequences--perhaps 100 bp's apart--and consequentially inactivate gene expression of the targeted promoter.

The bZIP Motif: Biology

A. Stable cell lines

Initial attempts at making stable cell lines expressing an inducible CEBP dominant-negative have been frustrating. Initially, the epitope tag was between the dominant-negative and the inducible estrogen binding domain in the primary sequence. Placing the epitope at the N-terminus of the protein allows for much easier detection of expression of our dominant-negative. The importance of having a stable cell system to examine the cellular DNA binding properties, i.e. in vivo footprints, of our inducible system demands that we continue in our attempts to make stable cell lines.

B. Transgenic mice

We have initiated collaborations with NIH researchers to construct transgenic mice using our designed dominant-negative proteins. An obvious question is whether the designed proteins will create a genetic phenotype. The clear possibility of creating a short lived dominant lethal with the designed dominant-negative protein influences the type of transgenic mice to be produced. We wish to examine the consequences of expression of the dominant-negatives using different viral promoters. Collaborators are interested in expression of these designed constructs in particular tissues--typically under the expression of promoter sequences under investigation in the respective laboratories. Dr. Arhneiter's group studies coat color. The disruption of the melanocytes critical for coat color is not expected to be lethal. CEBP is expressed in skin but it is not known if it is expressed in melanocytes. This system

may be a good simple phenotype to monitor the disruption of CEBP family function. Leslie Bruggeman and Paul Klotman examine kidney and skin, both tissues where CEBP is known to be expressed. Transgenic mice expressing our dominant-negative in these tissues could be illuminating. The generation of any biological phenotype would be welcome. Transgenic mice with phenotypes would allow our rationally designed dominant-negatives to be studied in a biologically relevant context.

C. Cellular localization of heterodimerizing leucine zipper

The ability to bring together two different proteins in the cell could be used for many cell biological experiments. In collaboration with Ken Yamada, we are currently attaching our heterodimerizing leucine zippers to a protein attached to the cell membrane and a second protein involved in cytoskeletal organization. This rearrangement of cellular protein under experimental control opens up a horizon of cell biological experiments.

Publications:

Thompson K, Vinson C, Freire E. (1993). Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transcription factor GCN4, Biochemistry 1993;32:5491-6.

Krylov D, Mikhailenko I, Vinson C. A thermodynamic scale for leucine zipper stability and specificity: **e** and **g** interhelical interactions. EMBO, in press.

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

PROJECT NUMBER
Z01 CB 05272-03 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

M. Mortin	Senior Staff Fellow	LB	NCI
L. Burke	BTP	LB	NCI
T. Jones	Biologist	LB	NCI
L. Kuhn	SRTP	LB	NCI
M. Park	Summer Student	LB	NCI
J. Huang	Volunteer	LB	NCI

COOPERATING UNITS (if any)
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.70	2.10	.60

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☐ (a1) Minors
☐ (a2) Interviews
B

SUMMARY OF WORK (Use standard unreduced 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 cloned in Drosophila and three have been mutated. The 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 functions or steps required for transcription by RNA polymerase II.

This year we have made progress in a number of our objectives to define the structure and function of RNA polymerase II. Much of our attention has been focused on defining the relationship between the suppressor domain in the second largest subunit with two domains in the largest subunit. We used in vitro mutagenesis to create a second largest subunit gene with both types of suppressor mutations. This gene does not appear to rescue null mutations in the second largest subunit but still behaves like a strong suppressor. This proves that the suppressor domain is essential and may provide a powerful method for saturating the largest subunit for mutations in interacting domains. We have also demonstrated that a potent inhibitor of RNA polymerase II elongation does not block the homeotic transformation caused by the altered functioning of specific RNA polymerase II mutations. This suggests that the defect occurs prior to the elongation step in transcription and that the in vitro transcription system we are establishing may help explain the cause of the Ubx effect.

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 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.

4. To determine the defect in transcription resulting in the Ubx effect.

Major Findings:

During the past year progress has been made in achieving all four of my outlined objectives. Sequence analysis described in last year's progress report defined a suppressor domain in the second-largest subunit of RNA polymerase II that interacts with two conserved domains in the largest subunit. All three identified domains were found to have weak sequence similarity to parts of DNA polymerase I comprising the DNA binding cleft. A description of these sequence and molecular modeling analyses has now been submitted for publication. I am in the process of testing a number of predictions made from the modeling analysis. First, I predict that the suppressor domain is an essential part of the RNA polymerase II enzyme yet no recessive lethal mutations in either Drosophila or yeast have been mapped to this region. PCR was used to synthesize a second-largest subunit gene containing amino acid substitutions found in both a strong and a mild suppressor. This construct was transformed back into the Drosophila germ line and is currently being tested. Preliminary observations indicate that this construct behaves like a strong suppressor in its ability to rescue one conditional lethal allele in the largest subunit, does not behave like a mild suppressor in that it cannot rescue a second conditional lethal allele normally rescued by mild suppressor mutations, and surprisingly does not rescue loss of function alleles in the second-largest subunit, as do both strong and mild suppressor mutations alone. This confirms that the suppressor domain is an essential part of the enzyme. The molecular modeling also predicts that any other interacting mutations in the two largest subunits will map within conserved domains that fall within regions of sequence similarity to the DNA binding cleft of DNA polymerase I. We have recently identified two new interacting mutations in the largest subunit and are in the process of sequencing them to see if this prediction is born out.

The completion of the cloning of the S3 suppressor mutation locus has proven to be more difficult than we had thought last year at this time. We have been unable to clone the 16kb genomic DNA surrounding the RNA encoding region thought to be the S3 locus into a transformation vector. We successfully subcloned a smaller 6.4kb genomic fragment, also thought to contain the entire transcribed region, into a transformation vector and obtained one stably transformed line of Drosophila; however, this fragment did not rescue the S3 locus. We are currently trying different genomic clones containing most of the original 16kb of genomic DNA. This is being combined with a more concerted effect to identify molecular markers that will better define the precise limits of the S3 locus. Cloning of the S8 suppressor locus has been begun by using PCR to clone an RNA binding gene that maps to the same region and is a candidate for the correct gene.

We are now able to make extracts from Drosophila embryos that will carry out an in vitro transcription reaction on defined templates that we will use to test Ubx activation and repression. Our next goal is to demonstrate that our system does respond correctly to the Ubx transcription factor. We will then be in a position to start testing extracts made from our mutant polymerase lines.

We have made a surprising observation concerning the enhancement, called the Ubx effect, by the transcription factor Ubx acting on specific RNA polymerase II mutations. The polymerase alleles that cause the Ubx effect are altered in their functioning, yet levels of the inhibitor a-amanitin that inactivate it do not block this enhancement. We have demonstrated that a-amanitin is taken up by the transformed tissue and that the intracellular concentration is sufficient to inhibit transcription. These observations suggest that the Ubx effect occurs prior to the block in transcription caused by the drug, that is prior to the elongation step. These results are currently being written up for publication.

Publications:

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;134:517-29.

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;13:4214-22.

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

PROJECT NUMBER
Z01 CB 05273-03 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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
C. Patterson	Pre-doctoral IRTA	LB	NCI

COOPERATING UNITS (if any)
Pablo Gejman, Clinical Genetics Branch, NIMH; Jonathan Benjamin, Clinical Neuroendocrinology Branch.

LAB/BRANCH
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Gene Structure and Regulation Section

INSTITUTE AND LOCATION
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.75	5.75	1.00

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☐ (a1) Minors
☒ (a2) Interviews

B

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

Methods and resources for the identification of genes involved in complex human characteristics and diseases are being assembled, tested and implemented. Attributes under study include sexual orientation in both males and females, psychological traits, susceptibility to alcoholism and substance abuse, and HIV progression.

Project Description

Objective: To identify genes that contribute to complex human characteristics and diseases.

Major Findings

A. Overview

Human beings are variable in both their genotype and phenotype. Due to the more than 3 million polymorphisms in the human genome, there are no two individuals (except identical twins) who have exactly the same genetic information. People also differ in their physical, mental, behavioral and psychological attributes and their susceptibility to various diseases. Although there is evidence that many such complex characteristics are influenced by heredity, the evidence is equivocal because few of the individual genes have been isolated.

The goal of our project is to develop and implement methods for identifying discrete genes that influence complex human characteristics and diseases. The search for such genes is difficult for several reasons: i) many different genes may be involved, either interactively or independently, so the effect of any single gene is likely to be small; ii) the environment, both biological and non-biological, may also play a significant role; and iii) different combinations of genes may have different effects under different conditions.

Our approach is sib-pair linkage analysis. The basic principle is that if a gene influences a trait, then siblings who share the same allele of that gene are more likely to be similar for the trait than are siblings who have different alleles. It follows that siblings who share the chromosomal region in which that gene resides will be more similar for the trait than siblings who have inherited different regions. Therefore, if we measure trait similarity as a function of polymorphic DNA marker identity in a population of sib-pairs, we can map trait genes to chromosomal loci.

There are several advantages to the sib-pair linkage approach. First, linkage analysis scans the genome in relatively large blocks of several million base-pairs (1 - 5 cM). Since none of the traits that we are studying have good "candidate genes" associated with them, we think it is wise to cast our net as broadly as possible. Second, sib-pair analysis is nonparametric; that is, it does not require prior knowledge of the frequency, penetrance, or transmission mode of the relevant genes. Finally, for traits that are influenced by multiple interacting genes or by common family environments, it is best to study closely related relatives such as siblings rather than extended families. Lastly, because our approach uses populations of families rather than individual families, it is likely to detect genes of broad rather than narrow influence.

Our strategy depends on the availability of highly informative, closely spaced DNA markers that span the human genome. Fortunately, such markers are now available for every chromosome through the efforts of the Human Genome Project. We have established semi-automated methods for the PCR-based detection and mapping of these markers on large numbers of human DNA samples.

We are applying this methodology to the genetic characterization of a number of complex traits and diseases that are described below.

B. Sexual Orientation

Sexual orientation is variable both between individuals and between genders. Last year, we reported family pedigree and DNA linkage studies indicating that at least some of the variability in sexual orientation in men is correlated with a genetic locus on the X-chromosome. Specifically, we found that homosexual men had more gay male relatives on the maternal than on the paternal side of the family, and that within a highly selected population of 40 homosexual brothers there was excess sharing of DNA markers from region Xq28.

We are currently attempting to replicate and extend this finding by several approaches:

i) Recruiting a second, independent set of gay male sib-pair families. We have currently completed the interviews on 28 such families and have additional families scheduled.

ii) Determining the genotypes of the heterosexual brothers of the Xq28-concordant sib-pairs. We've tested 3 such brothers to date, and each had the opposite Xq28 region as the homosexual siblings.

iii) Testing additional Xq28 markers in the hope of finding a linkage disequilibrium that will allow us to more precisely map the position of the putative sexual orientation locus. At this point we have not detected any significant allelic associations, but we have not yet assayed any markers in the interesting region of X-Y homology at the end of the Xq28 region. We hope to develop such markers in the near future.

We have also initiated a complementary study of sexual orientation in women. Family histories of sexual orientation were collected from 252 female probands: 62 women who identified as heterosexual, 113 women who were bisexual, and 177 lesbians. A preliminary analysis of the data indicates a significant familial clustering of homosexual orientation in these families. For example, the sister of a lesbian proband was 7-fold more likely to identify as non-heterosexual than the sister of a heterosexual index subject. Surprisingly, however, the rate of homosexuality was even higher among the daughters of lesbians, which is not expected from any simple genetic model. Furthermore, among second and third degree relatives, rates of homosexuality were elevated only in paternal cousins through an uncle. These data cannot distinguish between environmental and inherited sources of familiality.

To more definitively address the role of genes in female sexual orientation, a sib-pair linkage analysis has been initiated. We have completed interviews and collected DNA from 42 families in which there are two lesbian sisters and are currently analyzing this material with markers on the X chromosomes and on chromosomes 19 and 17.

C. Psychological Traits

Many psychological traits display a substantial degree of heritability in twin studies, but none of the contributing genes have been identified. Recently Dr. Jon Benjamin detected a weak correlation between the psychological trait of "self-reliance" and the color-vision locus at Xq28 -- the same region at which we mapped male sexual orientation. This coincidence has prompted us to initiate a linkage analysis of personality traits and the X chromosome.

The basic strategy is to administer personality questionnaires to brothers, map their X chromosomes with polymorphic markers, and correlate the phenotypic to genotypic sharing. With this aim, we have given the Cattell 16-PF and NEO-PI personality inventories to all of the brothers in our male sexual orientation study. In addition, we are collaborating with Dr. Benjamin to map the DNA of a set of randomly ascertained, predominantly heterosexual brothers that he is collecting at NIMH. The goal is to map approximately 500 pairs of brothers, which should give sufficient power to detect X-linked genes with effect sizes of as little as 10 percent.

D. Alcoholism

Alcohol abuse is an extremely serious medical problem. Although alcoholism is substantially familial, no relevant genes have been unequivocally identified. Several groups have claimed an association between alcoholism, polysubstance abuse and cocaine use and the DRD2/TaqI allele A1, which is located more than 10,000 basepairs downstream of the coding sequences of the gene for the dopamine D2 receptor. However, other groups, including our own, failed to detect any linkage or correlation between the DRD2 gene and addictive disorders. To more definitely address this problem, Drs. Pablo Gejman, Elliot Gershon and associates performed a detailed analysis of the DRD2 coding sequences by denaturing gradient gel electrophoresis and direct DNA sequencing. We provided Dr. Gejman with DNA samples from a series of homosexual male and female subjects who were recruited through the Whitman Walker Alcohol and Substance Abuse outpatient program and the NIAAA inpatient alcoholism program; each of these subjects had a history of alcohol abuse and one or more relatives with alcohol and/or drug addiction. No significant coding sequence mutations were found in any of the samples tested.

We collect information on alcohol and substance use for all participants in our linkage studies, including both the NIH and NIMH populations. As our genomic linkage scan proceeds, we will attempt to identify more promising loci for alcohol and substance abuse.

E. HIV/AIDS

One of the most striking features of the AIDS epidemic is individual variability in response to HIV infection. Some individuals become immunodeficient and develop cancer or opportunistic infections shortly following seroconversion whereas other individuals have remained healthy for 10 or more years following exposure to the virus. Research suggests that some of this variability may be due to host genes, but to date only a few dozen of the estimated 100,000 genes in the human genome have been tested for their role in HIV progression. Perhaps one of these genes holds the key to a completely new approach to AIDS treatment.

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. Several recent papers have shown an association between HLA haplotype and susceptibility to Kaposi's sarcoma. However, because these studies were performed on unrelated individuals, it is difficult to say if the observed differences were due to inheritance or population stratification. Our plan is to apply the sib-pair method to test the HLA locus and other chromosomal regions for linkage to HIV outcome. Through our studies of male sexual orientation, we have obtained several families with two HIV-positive brothers, and have tested these with over 40 polymorphic DNA markers. However, as might be expected from the small sample size, no significant correlations have been observed. We are currently making a focused effort to obtain more pairs of HIV-positive brothers, particularly those with Kaposi's sarcoma.

Publications:

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 Hum Genet 1993;53:844-52.

Hamer D, Hu S, Magnuson VL, Hu N, Pattatucci AML. A linkage between DNA markers on the X chromosome and male sexual orientation, Science 1993;261:321-7.

Gejman PV, Ram A, Gelernter J, Friedman E, Cao Q, Pickar D, Blum K, Noble EP, Kranzler HR, O'Malley S. Hamer DH, Whitsitt F, Rao P, DeLisi LE, Virkkunen M, Linnoila M, Goldman D, Gershon ES. No structural mutation in the dopamine D2 receptor gene in alcoholism or schizophrenia, JAMA 1994;271:204-8.

LeVay S, Hamer DH. Evidence for a biological influence in male homosexuality, Sci Am 1994;270:44-49.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05274-03 LB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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,

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

COOPERATING UNITS (if any)

Dr. W.N. Frankel, The Jackson Laboratory; Dr. M. Potter, Laboratory of Genetics, NCI; Dr. C. Kozak, Laboratory of Molecular Microbiology, NIAID

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

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.)

IAP oligonucleotide probes derived from expressed IAP elements detect restriction fragments which have characteristic strain distribution patters (SDPs) among different strains of mice, making them suitable multilocus probes for genome mapping. IAP proviral elements were mapped in an interspecific backcross between C57BL/6J and an inbred line of Mus spretus, SPRET/Ei, in collaboration with Wayne Frankel, The Jackson Laboratory. The map includes 51 IAP loci that have not been previously mapped and 23 IAP proviruses that had been previously mapped in recombinant inbred strains. This backcross panel is available as a community genetic mapping resource.

Inbred strains of mice differ in diabetes-susceptibility, and IAP gene expression in pancreatic B-cells has been correlated with susceptibility. Genomic DNAs have been analyzed with the IAP oligonucleotide probes to define genetic differences between diabetes-susceptible strains (C57BL/Ks and DBA/2) and a resistant strain (C57BL/6). Twenty-nine IAP loci were found to differ between the C57BL/6 and C57BL/Ks strains. A number of these loci mapped to chromosomal regions in which genes that play a role in diabetes are located. The results showed that a significant genetic contribution from a DBA/2-like strain is present in the C57BL/Ks strain.

Mouse plasmacytomas provide a model for several human B cell neoplasms. Tumor development in the susceptible BALB/c strain is determined by multiple genes, that have been studied in crosses between BALB/c and the resistant DBA/2 strain. Genomic mapping in the crosses with IAP oligonucleotide probes identified three IAP loci that are closely linked to the susceptibility phenotype on distal mouse chromosome 4.

Project Description

Objective:

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

Major Findings:

Last year we described work in which we determined chromosomal locations for IAP proviral loci, defined by sequences found in IAP elements expressed in LPS-stimulated lymphocytes (lymphocyte specific or LS elements) or in plasmacytomas. In *Hind*III digests, these IAP oligonucleotide probes each reacted with a limited number of restriction fragments that represent junctions between proviral and flanking DNA. These proviral elements were mapped in an interspecific backcross between C57BL/6J and an inbred strain of *Mus spretus*, SPRET/Ei in collaboration with Dr. Wayne Frankel, The Jackson Laboratory. This panel of backcross animals has been established as a resource for the mouse genome mapping community. The rate of polymorphism between markers in the inbred strains and *Mus spretus* exceeds 80% because of the evolutionary distance between them. We have assigned chromosomal locations to 74 IAP proviral loci. The map includes 51 IAP loci that have not been previously mapped and 23 IAP proviruses that we previously mapped in recombinant inbred (RI) strains. Comparable map positions were obtained for the IAP markers in the interspecific backcross and the RI strains. The mapped IAP loci were widely dispersed on the X chromosome (Chr) and all the autosomes except Chrs 9 and 19, providing useful genetic markers for linkage studies.

IAP proviral genes are constitutively expressed in pancreatic β-cells of certain diabetes-susceptible inbred mouse strains such as C57BL/KsJ (BKs) and DBA/2J (D), but not in β-cells of resistant strains such as C57BL/6J (B6). It was assumed that IAP expression in BKs reflected differences in location of a few IAP elements in this strain compared with the closely related diabetes-resistant B6 strain. Recently, preliminary results from this laboratory and studies at the Jackson Laboratory showed that substantial genetic differences exist between BKs and B6 mice. A survey of IAP and GLN proviral loci has now revealed that a total of 29 loci differ between the BKs, B6, and D strains. Our previous mapping in the BXD RI strains and the interspecific backcross described above, permitted us to identify the genomic locations of the regions that differed. It is now clear that the BKs and B6 strains are genetically much less closely related than had been assumed. Many of the genetic differences are consistent with the presence in the BKs strain of chromosomal segments derived from a DBA/2-like strain. These include regions mapped to Chrs 1, 3, 4, 5, 6, 7, 11, 12, 14, 15, and 17. A number of the IAP loci that differed between the diabetes-susceptible BKs and the diabetes-resistant B6 strains mapped to regions that contain genes that play a role in diabetes. These include the glucokinase gene (Gk) and insulin dependent diabetes susceptibility gene-4 (Idd4) on Chr 11, and the insulin-like growth factor gene (Igf2) and insulin-2 gene (Ins2) on Chr 7.

An LS1 provirus, designated Iapls1-10, mapped to distal Chr 4, in a region previously determined by Dr. Beverly Mock, Laboratory of Genetics, NCI, to be the location of two genes conferring susceptibility/resistance to plasmacytoma induction. Other IAP oligonucleotide probes were used to screen DNAs from progeny of crosses between the plasmacytoma susceptible BALB/c strain and the resistant DBA/2 strain, in collaboration with Dr. Michael Potter, Laboratory of Genetics, NCI. Two additional IAP loci that segregated with the susceptible phenotype have been identified and mapped to distal Chr 4.

Somatic cell hybrid panels consisting of cell lines containing limited numbers of BALB/c mouse chromosomes were used to assign chromosomal locations to BALB/c IAP loci in collaboration with Christine Kozak, Laboratory of Molecular Microbiology, NIAID. This has allowed us to identify chromosomal regions that are hypomethylated in plasmacytomas and other B-cell tumors in this strain of mouse (see Lueders and Kuff AR Z01 CB 05264-13 LB). At the present time there are no suitable RI sets or backcrosses for mapping genes in the BALB/c strain.

Publications:

Lueders KK, Frankel WN. Mapping of mouse intracisternal A-particle proviral markers in an interspecific backcross, Mammalian Genome, in press.

Lueders KK. Differences in intracisternal A-particle and GLN proviral loci suggest a genetic contribution from a DBA/2-like strain in generation of the C57BLKs strain, Mammalian Genome, in press.

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

PROJECT NUMBER
Z01 CB 08212-20 LB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

S. L. Berger	Chief, Genes and Gene Products Section	LB NCI
H.Y. Lee	Visiting Fellow	LB NCI
P. Mol	Visiting Fellow	LB NCI

COOPERATING UNITS (if any)

None

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SECTION
Genes and Gene Products Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	

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 new class of restriction endonucleases called hapaxoterministic enzymes or hapaxomers has been defined. These enzymes cut DNA outside of the recognition site or within an interrupted palindrome at bases which are not specified producing fragments with asymmetric, staggered ends. Upon ligation, these fragments are able to reassemble efficiently into the original structure. The ability to reunite fragments which were once contiguous means that hapaxomers cleaving DNA at many locations are virtually equivalent to restriction enzymes cutting at unique sites. The process is equivalent to putting Humpty Dumpty together again and has many applications.

The regulation of the prothymosin alpha gene was investigated in mouse L cells using constructs composed of the chloramphenicol acetyltransferase (CAT) gene fused to a series of prothymosin alpha promoter sequences ranging from 0.25 to 4.5 kb upstream of the beginning of transcription. Each was designed with and without an insertion of intron 1 of the prothymosin alpha gene positioned between the stop codon of CAT and the polyadenylation signal. All prothymosin alpha promoters were extremely potent. The presence of the intron located in cis reduced transcription about 5-fold, but in trans had no effect on CAT activity. The effect of normal and mutant myc and max proteins on transcription of the prothymosin alpha gene was investigated in cotransfection experiments. Despite the fact that normal myc and max bind as heterodimers in vitro to two E boxes located 1.2 kb upstream of the beginning of transcription and within intron 1, respectively, neither the normal nor the mutant proteins affected CAT activity in vivo. Disruption of the E boxes also failed to alter transcription. Even a dominant negative mutant of max which sequesters endogenous myc was unable to modulate transcription from the prothymosin alpha promoter. We conclude that myc does not regulate transcription of the prothymosin alpha gene in the general case and that reports by others to the contrary must be reevaluated.

The product of the prothymosin alpha gene is a protein which is phosphorylated throughout the cell cycle. The hapaxomer based method of generating mutants was used to map the location of the phosphates. Depending upon the mutation, phosphorylated amino acids were found at several locations within the prothymosin alpha protein. The phosphate appeared to be capable of migrating. Such behavior is unusual and does not fit current models of signal transduction.

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. This protein is required for survival. We plan to elucidate the function of prothymosin alpha.

Major Findings:

In order to study the regulation of the prothymosin alpha gene and the function of the phosphorylated protein product, it was necessary to generate mutants in the coding and noncoding regions of the gene, the introns, and the flanking regions containing sequences required for transcription. In some cases, the fusions had to be indiscernible—the introduction of exogenous sites for cleavage with restriction enzymes was contraindicated. We have developed a simple and novel method for producing such mutants. It can be applied to a wide variety of mutagenesis experiments including site specific mutagenesis, the addition or deletion of sequences, the isolation of large fragments that lack unique restriction sites, and the shuffling of introns and exons. The method makes use of a class of enzymes which we have named hapaxoterministic enzymes, or hapaxomers for short. These enzymes are derived from the existing categories of restriction enzymes usually designated type II, those with an element of symmetry in the recognition site, and type IIs, those whose recognition site lacks symmetry. However, the definition of the new class of enzymes does not depend on the nature of the recognition site. Rather it focuses on important features of the cleavage site. Hapaxomers are those restriction endonucleases which generate fragments with unique ends in a subset of DNA. The name is derived from hapaxo, a word employed by scholars of classical Greek to describe unique words, phrases, ideas, or textual features while keeping in mind the possibility that newly discovered documents might render the "unique" aspect no longer exceptional. So, too, hapaxoterministic refers to unique ends in a subset of DNA under consideration—be it a gene, plasmid, or genome— and not to the totality of DNA. Hapaxomers are drawn from enzymes whose recognition sites are interrupted "palindromes" or enzymes which cleave outside their recognition sites; only those which generate asymmetric, staggered ends with three or more unpaired bases are usually included. Upon ligation of a hapaxomeric collection of fragments, the original structure will be efficiently reconstituted. When bacteria were transformed with an 8 kb plasmid which had been cleaved into four fragments with Pfl MI and ligated, the number of colonies exceeded those obtained using the same plasmid cleaved at a unique Hind III site. Furthermore, all of the clones examined were perfect. By replacing normal fragments with mutant fragments bearing the correct termini, mutant plasmids or genes can easily be generated. In a different type of experiment, hapaxomeric sites were included in the primers used for the polymerase chain reaction. Because cleavage occurred at a distance from the recognition site in these examples, cutting within the primer not only produced the asymmetric staggered ends needed for efficient assembly, but also excised the site. Ligation of two such fragments resulted in a seamless fusion of two sequences. These experiments illustrate a simple principle: Symmetry equals competition among fragments for ligation partners and competition begets byproducts. By eliminating

symmetry at the termini, fragments can neither join to themselves nor to promiscuous partners. The at many locations are virtually equivalent to restriction enzymes cutting at unique sites.

In 1991 Eilers and Bishop reported that the myc proto oncogene protein increased transcription of the prothymosin alpha gene in the absence of protein synthesis suggesting that myc might bind directly to the prothymosin promoter. To study the regulation of the prothymosin alpha gene, we constructed and acquired plasmids containing myc and max. Two such constructs were expressed in Escherichia coli. The engineered genes contained the basic region and the helix-loop-helix and leucine zipper domains necessary for binding to DNA and for oligomerization, respectively. Exogenous sequences coding for carboxyl terminal histidine tails which facilitated purification of the encoded protein by means of nickel chelate column chromatography were also introduced. In addition, the two coding regions were truncated: the max gene lacked codons for the first eight amino acids; whereas the myc gene lacked sequences for amino acids 1-342, which include the transactivation domain.

We began with an in vitro investigation of two regions of the prothymosin alpha gene containing consensus E box sequences: a tract located 1.2 kb upstream of the beginning of transcription and a domain found in intron 1. Using labeled oligomers containing 26 bp identical to each of these regions, we demonstrated the following: that the myc protein alone could not shift either oligomer; that max alone resulted in weak signals; and that myc mixed with max resulted in a band shift with both oligomers. The shifted complexes were greatly reduced in intensity when cold, identical competitor DNA was included, but were unaffected by the addition of nonspecific DNA in which the central C and G residues of the E box were reversed. The data suggest that myc and max, probably as heterodimers, bind to the prothymosin alpha gene in vitro in two locations.

To determine whether the in vitro results occurred in vivo, it was necessary to design experiments which would be interpretable despite the presence of an endogenous myc gene. Toward this end, additional myc mutants were obtained from Dr. C. V. Dang of The Johns Hopkins University: a dominant negative myc in which the transactivation domain (amino acids 106-143) was deleted and a recessive negative myc lacking the helix-loop-helix domain (amino acids 371-412). A wild type gene driven by a mouse leukemia virus promoter was also acquired. The ability of these proteins to affect transcription of the prothymosin gene in mouse L cells was measured using constructs composed of the gene for chloramphenicol acetyltransferase (CAT) fused to a series of prothymosin alpha promoter sequences from 0.25 to 4.5 kb upstream of the beginning of transcription. Each of these CAT constructs was prepared with and without an insertion of intron 1 positioned between the translational stop codon of CAT and the polyadenylation signal. This location mimicked that of intron 1 in the wild type prothymosin alpha gene. Transfection of the CAT constructs lacking intron 1 revealed that the prothymosin alpha promoter, whether truncated to 250 bp or expanded to encompass 4.5 kb from the beginning of transcription was extremely potent. The presence of the intron reduced transcription about 5-fold in all cases when introduced in cis, as noted above, but had no effect in trans. The effect of normal and mutant myc proteins on the CAT construct containing 4.5 kb of the promoter in the presence and absence of intron 1 was investigated by cotransfecting myc together with a CAT construct into the L cells. Our results indicate that the presence of the recessive negative mutant myc gene, or the dominant negative mutant myc gene, or the wild type myc gene had no effect on CAT activity regardless of which prothymosin alpha specific sequences were nearby. However, since the endogenous myc gene was presumably transcribed, there may have been sufficient normal myc to support maximum transcription from a prothymosin promoter regardless of the added genes and their products.

A second approach targeted the E boxes. Since myc and max bind to these sequences in vitro, we explored their importance in vivo. Both E box sequences include Pml I sites that can be cleaved and

filled in to obliterate specific interactions with myc and max. Accordingly, the largest prothymosin alpha-CAT fusions one with and one without the intron were generated with disrupted E boxes. When each member of the pair was transfected into L cells, we were unable to discern the difference between transcription driven by the normal sequences and their disrupted equivalents. The conclusion was drawn that binding of myc and max to E box sequences in the promoter or in the intron is not necessary for maximum transcription of the prothymosin alpha gene.

A third approach made use of a dominant negative mutant of max designed to trap all the myc protein in the cell in unproductive complexes. In this mutant, called dMAX, the basic region (amino acids 1-28) was omitted. dMAX binds to myc, but the heterodimer is unable to bind DNA. Overexpression of the dMAX gene using a thymidine kinase promoter should result in the production of levels of dMAX high enough to sequester endogenous myc so that free myc will be unavailable for a regulatory role. When this mutant max gene was cotransfected into L cells together with either of the pair of CAT constructs bearing 4.5 kb of the prothymosin alpha promoter, we found no effect on CAT activity. None of our results support a vital role for myc in the transcription of the prothymosin alpha gene in L cells.

The product of the prothymosin alpha gene is a protein which is phosphorylated throughout the cell cycle. The hapaxomer based method of generating mutants was used to advantage in mapping the location of the phosphorylated amino acids. Earlier, we found that two percent of the protein was phosphorylated in vivo on the N-terminal acetylserine residue. To assess the importance of this residue, a mutant gene was prepared in which the codon for the serine at the N-terminus was replaced by an alanine. When transfected into COS cells and overexpressed, the cells continued to phosphorylate prothymosin alpha. However, most of the phosphate appeared not in the N-terminal Lys-C peptide, where it had been initially, but in an internal peptide which contains both a serine and a threonine residue. (There are no tyrosine residues in the protein.) Mutation of these residues singly or together to alanine residues did not abrogate phosphorylation. A triple mutant in which Ser 1 and 83 and Thr 85 were replaced by Ala was examined in detail. When the gene was transfected into COS cells, the resultant protein product was phosphorylated, but in this case the phosphate appeared in a peptide flanking the nuclear localization signal: the peptide contains a threonine residue. In this case, the mutant protein was clearly distinguished from the wild type because it was engineered to contain six histidine residues at the carboxyl terminus. Although the results cannot be unequivocally interpreted, they are consistent with the migration of a phosphate from one or more high energy positions to a serine or threonine residue which provides stability. The unusual behavior of the phosphate suggests that the underlying processes may be worthy of intense scrutiny.

Publications

Manrow RE, Berger SL. GAG triplets as splice acceptors of last resort: an unusual form of alternative splicing in prothymosin a pre-mRNA, J Mol Biol 1993;234:281-8.

Berger SL, Manrow RE, Lee, H-Y. Phoenix mutagenesis: one-step reassembly of multiply cleaved plasmids with mixtures of mutant and wild-type fragments, Anal Biochem 1993;214:571-9.

SUMMARY REPORT

LABORATORY OF CELL BIOLOGY

DCBDC, NCI

October 1, 1993 to September 30, 1994

The Laboratory of Cell Biology (Michael M. Gottesman, Chief) consists of the Molecular Cell Genetics Section (Michael M. Gottesman, Chief) and the Chemistry Section (Ettore Appella, Chief). Research in the Laboratory of Cell Biology emphasizes 7 major project areas: 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 encapsidation of HIV RNA. Approximately 38 personnel working on these 7 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 anti-cancer drugs including natural products and cisplatin frequently show cross-resistance to many other 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 delineated one molecular basis of resistance to natural products resulting from expression of the *MDR*1 gene which encodes the 170,000 dalton P-glycoprotein (PGP), an energy-dependent multidrug efflux pump. Current studies are focusing on the mechanism of action of this pleiotropic transporter, and the use of vectors encoding the *MDR*1 gene for gene therapy of cancer and inborn errors of metabolism. Analysis of this multidrug transporter through photoaffinity labeling studies, mutational alterations, and kinetic studies of drug transport, has led to a model in which natural product hydrophobic drugs which interact with the transmembrane domains of PGP are removed directly from the plasma membrane. Two approaches are being used to determine more precisely the mechanism of action of the PGP pump: (1) Purification and reconstitution of PGP by Suresh V. Ambudkar (Johns Hopkins University School of Medicine); and (2) Expression of PGP in heterologous model systems. P-glycoprotein has been purified to near homogeneity. Drug-dependent ATPase activity and ATP-dependent transport of vinblastine can be demonstrated in reconstitution experiments. PGP has also been expressed in the yeast *Saccharomyces cerevisiae* where it is a fully active multidrug transporter. Retroviral vectors encoding the *MDR*1 gene can be used to confer multidrug-resistance on bone marrow of mice resulting in selective advantage of transduced bone marrow cells *in vivo* and might be useful during anti-cancer therapy to protect the bone marrow of human patients from the toxic effects of chemotherapy. New *MDR*1-based retroviral vectors which use an internal ribosome entry site to allow efficient expression of non-selectable genes have been constructed. This approach could allow selection of human bone marrow cells which express otherwise non-selectable genes such as those encoding glucocerebrosidase (Gaucher disease) or alpha galactosidase (Fabry disease).

Studies on p53

Under the supervision of Ettore Appella in the Chemistry Section the growth inhibitory effect of wild-type (wt) p53 is being studied. Expression of wt-p53, which has a unique conformation and phosphorylation state, induces G1-arrest and transactivates the WAF1/CIP1 gene that encodes the p21 protein, an inhibitor of cyclin-dependent kinases (CDK). Wt-p53 dependent G1-arrest is accompanied by decreased expression of the B-*myb* gene and high levels of ectopic B-*myb* expression rescues cells from p53 induced G1-arrest. The phosphorylation sites of p53 have been analyzed and DNA-PK, a nuclear protein kinase, has been shown to phosphorylate Ser at position 15 *in vivo*. Mutants in which the codon for Ser 15 has been changed to Ala are partially defective in their ability to block cell cycle

progression. However, human p53 mutants in which Ser 392 is changed to Ala or Asp are indistinguishable from wt-p53 in their ability to activate specific expression *in vivo* or *in vitro*.

Proteolysis

In the Molecular Cell Genetics Section, Michael R. Maurizi has been studying prokaryotic and mammalian ATP-dependent proteases at the molecular and cellular level. Together with Drs. Michael M. Gottesman and Nan Wang, the gene for a human homolog of the *E. coli* ATP-dependent Lon protease has been cloned, sequenced, and expressed in bacteria. The sequence of hLon shows about 41% identity to *E. coli* Lon, including the ATP-binding region and the proteolytic active site, and the cloned protein displays ATP-dependent proteolytic activity *in vitro*. hLon is located in the mitochondrial matrix. Subsequent studies in other laboratories have shown that hLon plays an important role in degradation of misfolded proteins and is essential for maintenance of functional mitochondria. The gene for the proteolytic component of another ATP-dependent protease, ClpP, has been cloned from a human cDNA library. The hClpP amino acid sequence is >60% identical to that of the bacterial protease, and the cloned protein expresses peptidase activity *in vitro*. Immunofluorescence localization and subcellular fractionation have also localized hClpP to human mitochondria.

Structural and biochemical studies with *E. coli* Clp protease have shown that the functional protease is a high molecular weight complex reminiscent of the eukaryotic 26S proteasome. Electron microscopic images show that two hexamers of ClpP subunits are superimposed to form a core which is flanked on each side by a hexameric ring of ClpA subunits. ClpA appears to have a bi-lobed structure, the lobes apparently corresponding to the two ATP-binding domains identified by amino acid sequence. Mutants of ClpA with amino acid substitutions in the ATP-binding site of the first domain are defective for formation of the ClpA hexamer, but their activity can be restored by formation of hybrids with the wild-type indicating the importance of cooperative subunit interactions for activity of ClpA. Mutations in the second ATP-binding site indicate its role in promoting conformational changes in ClpP and in the energy-requiring steps for presentation of substrates for degradation. Specific peptide substrates have been cross-linked to the allosteric substrate binding site of ClpA, which has been tentatively localized to the first domain.

In collaborative studies with Sue Wickner and Susan Gottesman, ClpA has been shown to display an ATP-dependent chaperone-like activity which alone can substitute *in vitro* for the classical chaperone system composed of DnaJ, DnaK, and GrpE. These results delineate at least two steps in the mechanism of ATP-dependent degradation, the first of which is an energy requiring unfolding reaction followed by another energy requiring step needed for presentation of the unfolded form of the substrate to ClpP.

Malignantly transformed cells secrete many proteases at increased levels, including the cysteine acid proteases cathepsins L and B. To elucidate the role of these proteases in normal physiology and in malignant cell growth and metastases, in work supervised by Michael M. Gottesman and Vincent J. Hearing, vectors for insertional mutagenesis of cathepsins L and B in B16 mouse melanoma cells and mouse embryonic stem (ES) cells have been constructed.

Melanoma and Melanogenesis

This project, directed by Vincent J. Hearing, is aimed at characterizing parameters critical to the growth and differentiation of melanocytes and their relevance to the growth and metastasis of transformed melanocytes (termed malignant melanoma). Several distinct melanogenic enzymes that interact to regulate the quality and quantity of pigment produced within melanocytes have been identified, isolated and characterized. These proteins are encoded by a family of pigmentation-related genes that are specifically expressed by mammalian melanocytes. Although expression of these genes is specific to pigment producing tissues, they are regulated independently following stimulation of

differentiation. The phenotypic properties of the melanins produced by these regulatory catalytic controls can differ dramatically, and their effects on the functional and photoprotective properties of melanins is being actively studied. Melanoma-specific antigens abnormally expressed by transformed melanocytes which play a role in the host's immune responses to tumor growth continue to be characterized. Monoclonal antibodies to one of those antigens, termed B700, specifically cross-react with human melanoma and have been useful as highly specific probes for detecting malignant melanoma. Intravenous treatment of tumor-bearing hosts with those antibodies provides significant protection against metastatic growth. B700 is the immunodominant antigen in a melanoma vaccine being developed that improves host survival in a novel model for spontaneous melanoma metastasis.

Antigen Processing and Presentation

Research supervised by Ettore Appella in the Chemistry Section has utilized mass spectrometry for the structural characterization of naturally processed peptides bound to Class I and Class II MHC molecules. Allele-specific peptide motifs for the human MHC Class I molecules HLA-A1, A3, A11, and A24 were defined. Nonamers were the predominant species, although peptides of 8, 10, 11, and 12 amino acids were also identified. The data suggest that different naturally occurring longer peptides can bind in different conformations to Class I MHC molecules and that peptide conformation, rather than sequence per se, plays an important role in determining the antigenic identity of the HLA-peptide complexes. By the use of synthetic peptide libraries the binding motifs recognized by DR and DQ Class II isotypes were analyzed. The data suggest that the two isotypes present different peptides, thus maximizing the repertoire of different epitopes available to helper T-cells.

Additional studies in the Chemistry Section have highlighted the role played by a new antigen of 118 amino acids which is expressed at high levels by human melanoma cells and in lower amounts by cultured melanocytes and retina. Nine of ten CTL lines restricted by HLA-A2.1 recognized transfectants expressing this antigen. Based on known HLA-A2.1 binding motifs, one nonamer peptide was identified to be most effective in sensitizing target cells for CTL lysis. This peptide, therefore, should prove to be very useful for the development of immunotherapeutic strategies.

Finally, a 19 nucleotide RNA from HIV-1, which contains a stem and loop structure, was defined as the minimal size of the RNA capable of binding the nucleocapsid viral protein NCp7. This binding site lies in a region highly conserved among different HIV-1 isolates and includes the major 5' splice junction. Studies of the complex of the 19-nucleotide RNA with the NCp7 by NMR will aid in the design of antiviral agents capable of inhibiting HIV-1 NCp7 functions and genomic RNA packaging.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03229-24 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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

B

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

Most CTLs recognize foreign antigens in the form of a peptide fragment bound to the MHC Class I molecule. Allele-specific peptide motifs for the human MHC Class I molecules, HLA-A1, A3, A11 and A24 were characterized by tandem mass spectrometry. Nonamers were the predominant species, although peptides of 8, 10, 11 and 12 amino acids in length were also identified. These peptides displayed anchor residues predicted by the specific motifs at position 2 and at the COOH-terminal, regardless of peptide length. An equilibrium binding assay was used to measure the affinities of naturally processed peptides of various lengths for the HLA-A2.1 molecule. The data suggest that different naturally occurring longer peptides can bind in different conformations to Class I MHC molecules. The ability of MHC molecules to accommodate the same peptide in different conformations appears to have distinct advantages to the immune system. An analysis of the constraints of the interaction of a natural peptide-HLA-A2.1 complex and the T-cell receptor was initiated. Data obtained thus far suggest that peptide conformation, rather than sequence per se, plays an important role in determining the antigenic identity of the HLA-A2.1-peptide complex. Class II MHC molecules play a major role in the immune response by binding peptide fragments of exogenous antigens and displaying them on the surface of antigen presenting cells to helper T-cells. By the combination of large libraries of synthetic peptides and of substitution and truncation analogues, the peptide binding motifs recognized by different Class II isotypes DR and DQ were analyzed. The data suggest that the two isotypes have evolved to present different peptides, thus maximizing the repertoire of different epitopes available to helper T-cells. Genetic differences that encode structural variation between Class II molecules are linked to susceptibility or resistance to several diseases. A model system has been developed to understand the molecular events that regulate antigen binding to Class II molecules, since self-peptide binding to particular Class II alleles may be important for initiation and/or maintenance of autoimmunity. Following incubation of various concentrations of a test peptide with live cells expressing single DR molecules or two or more DR and DQ molecules, we have detected *in vivo* competition between DR and DQ molecules for binding of this test peptide. The specific cellular compartment in which peptides bind to Class II molecules and the extent of competition between structurally different Class II molecules for binding of antigenic peptides are being defined.

Major Findings:

1. T-cell antigen recognition and major histocompatibility complex (MHC) antigens.

1) Antigen-specific T-cell responses require the presentation of processed peptides in association with polymorphic major histocompatibility complex (MHC) Class I and Class II glycoproteins. Most cytotoxic T-lymphocytes (CTLs) recognize foreign peptides in association with MHC Class I molecules. These antigenic peptides are derived by the degradation of various proteins endogenously synthesized by antigen presenting cells and are transported to the endoplasmic reticulum where they interact with Class I heavy chains to facilitate proper folding and association with β_2-microglobulin. These peptide - Class I complexes are then routed to the cell surface, where they can be scrutinized by T-cells. Knowledge of the molecular details of the peptide - Class I interaction derives from large amounts of recent data, including the three-dimensional structure of Class I molecules and of the peptide groove occupied by naturally processed peptides or single peptide epitopes. Class I-bound peptides are in general restricted in length to 8-10 amino acids and show peptide motifs that are allele-specific with up to three anchor positions. This is consistent with the multiple pockets and the close-ended structure of the MHC Class I peptide-binding groove.

We have analyzed in detail the peptide motifs of the most common human Class I molecules. This knowledge is important for the identification of potential CTL epitopes from the primary sequences of autoreactive or pathogen derived proteins. Our approach has involved direct amino acid sequencing of naturally processed peptides from affinity purified Class I molecules by tandem mass spectrometry and determination of the HLA binding capacity of peptide sequence motifs containing polyalanine analogs. For HLA-A1, A3, A11 and A24, nonapeptides were the predominant species, although peptides of 8, 10, 11, and 12 residues were also identified. All the naturally processed peptides sequenced were synthesized and shown to bind well to the appropriate HLA-A alleles. Thus, the knowledge of the specific motifs for the most frequent HLA alleles and the availability of quantitative Class I binding assays will aid in the search for potential CTL epitopes with clinical relevance.

Previous studies have suggested that the naturally processed peptides associated with most Class I molecules are predominantly 9 residues long. The predominance of 9-mer peptides reflects the unique stabilization provided by H-bond networks in the A and F pockets to the amino- and carboxyl-termini of peptides of this length. Later studies have suggested that longer peptides could be accommodated in the binding site, but this binding results in a kink in the central portion of the backbone. Our recent findings support this mode of binding. The presentation of a given peptide sequence in multiple lengths is likely to have a significant effect on immune recognition. The ability of MHC molecules to accommodate peptides longer than 9 residues, for example, may increase epitope complexity and allow an individual to be tolerant to a given peptide in one length and conformation and yet be fully responsive to the same peptide sequence presented in another length and conformation.

2) We previously isolated from immunoaffinity-purified HLA-A2.1 molecules of a human lymphoblastoid cell line a nonpeptide that in association with HLA-A2.1 is recognized by the antigen-specific receptor of a murine $CD8^+$ T-cell clone. A search of protein sequence data bases has not revealed this peptide in known proteins. The murine clone is analogous in its behavior to "alloreactive" T-cells largely responsible for the rejection of normal organ allografts.

Recent studies have indicated that, for MHC Class I molecules, peptides bind in a very similar conformation using conserved anchor residues, so that only three or four amino acid side chains of a peptide are accessible to the T-cell receptor (TCR). However, the complex nature of the interaction of peptide-MHC and TCR have made it difficult to discern what factors govern T-cell recognition and epitope specificity. We have initiated a detailed analysis of the constraints on recognition of the Class I

peptide epitope seen by the above murine T-cell clone by using a panel of peptide analogs. The conclusion drawn from our data is that recognition of the HLA-A2.1 peptide epitope by the CTL clone is not characterized by absolute requirements for specific amino acids at any of the nine peptide positions. While a number of conservative/semi-conservative substitutions are accommodated, recognition was highly sensitive to changes in the mid-region of the peptide. We are currently evaluating other T-cell clones restricted by HLA-A2.1 to see whether they show a similar pattern of recognition. A more thorough understanding of T-cell recognition will allow the generation of peptide partial agonists *or* antagonists in order to manipulate the immune system.

3) MHC Class II antigens bind peptides derived from both exogenous proteins and from secretory or integral membrane proteins that are synthesized by the antigen presenting cells. In contrast to peptides associated with MHC Class I, those associated with MHC Class II are typically 10-34 residues in length and have ragged amino and carboxyl termini. The ability of peptides to vary in length is consistent with the open-ended structure of the MHC Class II peptide-binding groove. Class II bound peptides also have allele-specific motifs which have been more difficult to characterize because of the considerable variation in peptide length. Crystallographic analysis of a Class II molecule (HLA-DRI) with a single peptide from the influenza virus haemagglutinin has shown that the peptide binds in a straight extended conformation. Pockets in the peptide-binding site accommodate five of the thirteen side chains of the bound portion of the peptide and explain the peptide specificity of HLA-DRI. Thus, in contrast to the peptide-binding motifs for MHC Class I, which allow only one or two amino acids as anchor residues, binding motifs for MHC Class II appear to have more loosely defined patterns. In contrast to the information available for DR, little is known about the interaction between peptide sequences and the other two human isotypes, DQ and DP. More information on the DQ molecules is desirable because of their relatively high expression and their association with several pathological states, such as juvenile diabetes, celiac disease and multiple sclerosis.

We have recently described a motif based on the use of large synthetic peptide libraries for DQ3.1. By this method we found that DQ3.1 molecules utilize a major anchor residue in position 5 that appears to have a small and hydrophobic side chain. The other positions investigated all appear to be very permissive, with only certain residues, such as proline and those with positive or negative charges, apparently not well tolerated. The motif recognized by DQ3.1 molecules is completely different from the one recognized by DR molecules. However, the DQ3.1 motif is remarkably similar to the one recognized by IA^d molecules, in which two small hydrophobic residues also acted as anchor residues, but with a different spacing. It is of further interest to note that the DQ molecules are the human evolutionary equivalent of IA molecules in the mouse.

Recently we have been interested in testing the hypothesis that different Class II molecules will compete for binding and presentation of a single peptide. Self peptide binding to particular Class II alleles may be important for initiation and/or maintenance of autoimmunity. Understanding the role of competitive peptide binding events in antigen presentation will be particularly important since humans are heterozygous, and multiple DR, DQ and DP molecules are codominantly expressed in antigen presenting cells. We have developed a model system for studying the formation of peptide-Class II complexes in live cells. This system is being used to determine if competition between different HLA Class II molecules for a single promiscuous peptide influences the number and kind of peptide-Class II complexes formed in the cell. Preliminary results indicate that there is *in vivo* competition between DR and DQ molecules. Future studies will include different types of antigen presenting cells to test the relevance of competitive binding interactions for antigen presentation in a complex molecular environment containing multiple Class II molecules.

II. Tumor Antigens

The immune response directed against chemically induced sarcomas has been attributed to T-cell mediated responses to tumor specific transplantation antigens (TSTA). Recently it has been reported that in a methyl-cholanthrene induced sarcoma (Meth A) the TSTA activity was associated with a Mr 110,000 glycoprotein, gp110. We have purified the gp110 to homogeneity and shown that an epitope on gp110 is recognized by a murine CTL clone restricted by H-2K^d. Our current research is focused on the molecular cloning of the gp110 cDNA for analyzing transfected target cells both for CTL reactivity and transplantation immunity. This information will then be utilized to define the antigenic polymorphism of gp110 expressed by other chemically induced tumors, as well as extend the analysis of gp110 to human tumors.

Lymphocytes of melanoma patients can be stimulated *in vitro* with autologous tumor cells to generate anti-tumor CTLs. Several groups have derived from HLA-A2 patients CTLs that lyse not only the autologous tumor cells but also a large proportion of the cell lines derived from the tumors of other HLA-A2 patients. Two normal self-proteins, MAGE-1 and tyrosinase have been identified as antigens recognized by melanoma-specific T-cells. Recently another self-protein, gp100, has been described as a third antigen recognized in the context of HLA-A2. This antigen may be particularly useful for the development of an active immunotherapy, since the administration of the corresponding CTL plus interleukin-2 resulted in the regression of metastatic cancer in the autologous patient. Another antigen of 118 amino acids has been identified that is expressed in most melanoma cells as well as cultured melanocytes and retina. Nine of ten CTLs lines restricted by HLA-A2.1 recognized transfectants expressing this antigen. Based on the known HLA-A2.1 binding motifs, we have synthesized 23 peptides in an attempt to identify the epitopes recognized by the CTLs. One 9-mer peptide was most effective in sensitizing target cells for CTL lysis. Therefore, this peptide is a very common immunogenic epitope for HLA-A2.1 restricted melanoma specific CTLs and may be very useful for the development of immunotherapeutic strategies. Intensive efforts are underway to ascertain if such a peptide plays a role in eliciting T-cell immune responses *in vitro* or *in vivo* and how effective these responses might be. The remarkable potential of this information for leading to effective therapy of melanomas ensures that much future work will be directed toward the identification of relevant peptide sequences in other types of cancer as well as infectious diseases and autoimmune disorders.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05597-05 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

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*)

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	S. K. Singh	Visiting Fellow	LCB, NCI
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	4.0	0.5

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☐ (a2) Interviews

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

In vivo, rapid degradation of regulatory proteins as well as the maintenance function of degrading abnormal proteins is energy-dependent. Our research has focused on the ATP-dependent Clp and Lon proteases from *E. coli* and their homologs in human cells. The gene for a human Lon protease (approximately 41 percent identity to *E. coli* Lon) has been cloned and expressed in bacteria. The sequence of hLon has an ATP-binding site and a proteolytic active site identical to *E. coli* Lon, and the cloned protein displays ATP-dependent proteolytic activity *in vitro*. Immunofluorescence and *in vitro* uptake studies have localized hLon to the mitochondrial matrix. The gene for a homolog of ClpP, the proteolytic component of Clp protease, has been cloned from a human cDNA library. The amino acid sequence of hClpP is greater than 60 percent identical to that of the bacterial protease, and the cloned protein is an active peptidase. hClpP also appears to be localized in mitochondria. Electron microscopic studies show that *E. coli* Clp protease has a structure analogous to that of the eukaryotic 26S proteasome. Two hexamers of ClpP subunits are superimposed to form the proteolytic core, and a hexamer of ClpA subunits is bound to each face of ClpP. The hexamer of ClpA appears to have a bi-lobed structure, possibly corresponding to the two ATP-binding domains identified by amino acid sequence. Mutants of ClpA with amino acid substitutions in the ATP-binding site of the first domain are defective for formation of the ClpA hexamer. Formation of hybrids between inactive mutants and wild-type ClpA restores activity to the mutant, indicating that cooperative subunit interactions affect the expression of ClpA activity. Mutations in the second domain ATP-binding site drastically decrease energy-dependent activation of proteolysis, without preventing interaction between ClpA and ClpP or activation of a limited peptidase activity of ClpP. Cross-linking of specific peptides to the allosteric substrate binding site of ClpA suggests that the initial interaction of protein substrates and Clp protease is with a site in the first domain of ClpA. In collaborative studies with Drs. S. Wickner and S. Gottesman, ClpA has been shown to have an ATP-dependent chaperone activity. ClpA promotes the dissociation of an inactive dimeric form of the DNA replication protein, RepA, into an active monomer. Release of RepA from its complex with ClpA requires ATP hydrolysis. When ClpP is present, ClpA promotes the rapid degradation of the RepA protein. These results delineate at least two steps in the mechanism of ATP-dependent degradation, the first of which is an energy requiring unfolding reaction followed by another energy requiring step to present the unfolded substrate to ClpP.

Major Findings:

(1) Dr. Mark Thompson has synthesized a series of peptide substrates based on the sequence, FAPHMALVPV, which are degraded by ClpP only in the presence of ClpA. ATP hydrolysis is not required for degradation of these peptides, indicating that nucleotide-promoted interaction between ClpA and ClpP produces a conformational change in ClpP allowing peptides, and presumably regions of proteins, better access to the active site of ClpP. Cleavage of FAPHMALVPV depends on initial interaction of the peptide with a binding site on ClpA. This site on ClpA is likely to be part of the active site on ClpA involved in unfolding and capture of potential targets for degradation. Protein substrates and propeptide compete at least partially for binding at this allosteric site, and binding of FAPHMALVPV is about 100-fold tighter there than at the protease active site. A photo-affinity derivative of FAPHMALVPV (with D-Met in place of L-Met) has been synthesized and cross-linked to ClpA. About 1 peptide was cross-liked per hexamer of ClpA. Incorporation of the peptide resulted in complete loss of activity of ClpA in promoting ClpP-dependent casein degradation, suggesting that FAPHMALVPV and proteins bind to the same site on ClpA. Digestion of ClpA after incorporation of the peptide allowed isolation of a peptide fragment of ClpA with the affinity ligand attached. The modified region of ClpA is located in the first domain. Additional derivatives of FAPHMALVPV with photo-activatable groups positioned to modify different portions of ClpA have been prepared and have been cross-linked to ClpA. Studies are underway to identify the sites of attachment of these probes in order to get a more complete map of the peptide binding site of ClpA.

(2) Dr. S. K. Singh has made site directed mutations by replacing the invariant lysine residue in the ATP consensus sequences in the two domains of ClpA and studied enzymatic and structural properties of the mutants and of hybrid molecules formed with mutant and wild-type ClpA proteins. Mutant proteins have lower affinity for nucleotide and drastically reduced ATPase activities at the mutated site. Activity that depends on nucleotide binding alone can be expressed at high concentrations of nucleotide, but activities dependent on ATP hydrolysis at one of the ATPase sites were defective in mutants altered at that site. Enzymatic properties of the mutants indicated that domain II of ClpA has a more active ATPase activity and that ATP hydrolysis at that site plays the major role in promoting ATP-dependent protein degradation. Domain II mutants can form hexamers and interact with ClpP to promote peptide degradation, which does not require ATP hydrolysis, but have <10% of the protein degradative activity of the wild-type enzyme. Specific domain I mutants (K220R and K220V) can assemble when ATP is in excess and can promote protein degradation ~70% as well as wild-type ClpA. The domain I mutant, K220Q, had the greatest defect in hexamer formation and association with ClpP. This mutant had no activity in promoting the proteolytic activity of ClpP and had no ATPase activity, confirming that assembly of the hexamer of ClpA is needed for expression of enzymatic activity by ClpA. K220Q was able to form mixed hexamers with wild-type ClpA. The basal ATPase activity of ClpA, measured in the absence of ClpP, was inhibited by interaction with K220Q. However, when ClpP was present, hybrids of wild-type and K220Q expressed ATPase activity and the ability to activate both peptide and protein degradation. The activities of the hybrids indicated that not only was the wild-type ClpA in the hybrids active but the mutant K220Q also expressed activity. Thus, interaction with ClpP must produce a further structural change in ClpA, promoting an active conformation in both wild-type and mutant subunits within the hexamer. Activation of the ATPase activity of K220Q in hybrids also suggests that ClpP interacts with domain II of ClpA.

(3) Electron microscopy studies of the multimeric complex of ClpAP have been done in collaboration with Dr. Martin Kessel (NIAMS). ClpP is arranged as two hexameric rings superimposed on each other. Mutant ClpP, which retains the 14 amino acid pro-peptide, has an appearance similar to wild-type ClpP, but the central hole in the hexameric rings is filled with protein, suggesting that the pro-peptide extensions are situated near the central axis of the molecule. In other zymogens, the pro-peptide occupies a groove that forms part of the substrate binding site of the protease. Thus, the substrate

binding sites and the active sites may be located near the central cavity in the particle. Computer enhanced image processing has been used to visualize negatively stained images of the complex of ClpA and ClpP. ClpA appears to have a bi-lobed structure, possibly corresponding to the two ATP-binding domains predicted by analysis of the primary structure of ClpA. The size of ClpA is consistent with a hexameric arrangement of subunits. The molecular weight of ClpA, determined by sedimentation equilibrium, was 500, 000, also consistent with a hexameric structure. An averaged image from 38 particles of ClpAP reveals an edge-on view of ClpP, flanked on each side by a hexamer of ClpA. The components are arranged so that the planes of the hexameric rings are parallel. Particles composed of dodecameric ClpP and a single hexamer of ClpA were observed when an excess of ClpP was present. It is possible that *in vivo*, ClpP can exist in association with either 1 or 2 hexamers of ClpA or that opposite faces of ClpP can be associated with ClpA and ClpX, another ATPase component that we have shown is able to activate specific protein degradation by ClpP *in vivo*.

(4) In collaboration with Drs. Sue Wickner and Susan Gottesman, we have recently shown that ClpA has an ATP-dependent chaperone-like activity. ClpA promotes unfolding of the P1 replication protein, RepA, converting it from an inactive dimer to an active monomer. RepA activated by ClpA has the same DNA binding activity as RepA activated by DnaJ and DnaK and requires ATP hydrolysis to drive the process. When ClpP is present with ClpA, RepA is rapidly degraded, also in an ATP-dependent process. Thus, the first step in the mechanism by which ClpA may activate proteolysis may involve binding to and promoting unfolding of a protein. Unfolding of the protein may result in release and spontaneous refolding of the protein or in retention of the unfolded protein on ClpA, followed by ATP-driven translocation of the unfolded protein into the active sites of ClpP. The two domains of ClpA might be expected to contribute one or the other of these activities. Current data suggests that domain I interacts first with the protein and domain II is involved with presenting the substrate to ClpP in an ATP-dependent process.

(5) Together with Drs. M. Gottesman and N. Wang and a pre-doctoral fellow, Leslie Emmert, the gene for a human homolog of the *E. coli* ATP-dependent Lon protease has been cloned, sequenced, and expressed in bacteria. The sequence of hLon shows about 41% identity to *E. coli* Lon including the ATP-binding region and the proteolytic active site, and the cloned protein displays ATP-dependent proteolytic activity *in vitro*. The hLon has been cloned with an epitope tag at the C-terminal end, and immunofluorescence studies using antibodies against the epitope have been used to demonstrate the mitochondrial location of the protein. *In vitro* synthesized hLon can be taken up into suspensions of purified rat liver mitochondria, where it is processed to a size similar to that of the mature protein in human mitochondria. The gene for the proteolytic component of another ATP-dependent protease, ClpP, has been cloned from a human cDNA library. hClpP amino acid sequence is >60% identical to the bacterial protease, and the cloned protein expresses peptidase activity *in vitro*. Immunofluorescence localization and sub-cellular fractionation have localized hClpP also to human mitochondria.

Publications:

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

PROJECT NUMBER
Z01 CB 05598-05 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
13.5	11.5	2.0

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

B

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

Our work has emphasized two aspects of the molecular biology of multidrug resistance (MDR) in cancer cells: 1) the mechanism of action of the multidrug transporter also known as P-glycoprotein (PGP), the product of the *MDR*1 gene; and 2) the use of vectors encoding the *MDR*1 gene to confer selective advantage on cultured cells and in cells in intact animals and patients. Mechanistic studies have involved biochemical purification of both naturally occurring (from MDR cell lines) and recombinant (from baculovirus) PGP. PGP purified to near homogeneity demonstrates drug-dependent ATPase activity and transport activity when reconstituted in lipid vesicles. Phosphorylation of PGP is not necessary for its activity, as shown by recombinant expression vectors in which all of the known phosphorylation sites on PGP have been deleted. A new kinase present in plasma membranes of cultured cells which phosphorylates PGP has been discovered and purified from cultured cells. To develop a model system for studying the effect of specific mutations on ATPase and substrate binding activities of PGP, the human *MDR*1 cDNA has been expressed in the yeast *Saccharomyces cerevisiae*, resulting in resistance to several different anti-cancer drugs. For gene therapy of cancers, *MDR*1-encoding retroviral vectors which confer MDR on the bone marrow of mice have been developed. One bicistronic vector in which the Herpes simplex virus thymidine kinase (HSK-TK) gene is under control of an internal ribosome entry site (IRES) present on the same mRNA which encodes *MDR*1 can be used to kill cancer cells which have been inadvertently transduced with the *MDR*1 gene during gene therapy to protect bone marrow against the toxic effects of chemotherapy. Other vectors for gene therapy of inborn errors of metabolism carry the *MDR*1 cDNA as a selectable marker and the non-selectable cDNAs for glucocerebrosidase (Gaucher disease), alpha galactosidase (Fabry disease) and the 91 kDa subunit of the NADPH oxidase complex (chronic granulomatous disease).

Other Professional Personnel:

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B. Ni	Visiting Fellow	LCB, NCI
S. Goldenberg	Microbiologist	LCB, NCI
D.-W. Shen	Visiting Associate	LCB, NCI
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Major Findings:

1. Reconstitution of transport with purified PGP: P-glycoprotein (PGP) has been purified to near homogeneity from highly multidrug resistant (MDR) KB-V1 cells after extraction with octyl glucoside and fractionation on DEAE Sepharose CL6-B and wheat germ agglutinin columns. This purified protein in detergent shows some basal ATPase activity, but when reconstituted into liposomes prepared from *E. coli* lipids, cholesterol, phosphatidylserine and phosphatidylcholines, displays both basal and drug-stimulated ATPase activity (15-30 mmole of phosphate hydrolyzed per mg of protein). When reconstitution is performed under conditions which allow formation of proteoliposomes with reduced lipid to protein ratio, ATP-dependent transport of vinblastine can be clearly demonstrated. These studies provide biochemical evidence that PGP itself is sufficient for drug transport. To obtain larger amounts of material suitable for structural analysis and analysis of the mechanism of action of PGP, the human *MDR*1 cDNA was engineered to include a nucleotide encoding a polyHis (6) track at the carboxy-terminus or in the linker region connecting the two homologous halves, and inserted into a baculovirus vector. Protein encoded by the baculovirus vector with the polyHis track at its carboxy-terminus, expressed in insect Sf9 cells, demonstrates drug-dependent ATPase activity in crude membrane preparations. PGP-His(6) can be purified on Ni-NTA columns.

2. Phosphorylation is not essential for function of PGP: Indirect evidence has suggested that phosphorylation of PGP by protein kinase C (PKC) may activate its transport function. There are five sites of phosphorylation by PKC in PGP in the region connecting the two halves of PGP (Ser 661, Ser 667, Ser 671, Ser 675, Ser 683), of which three (Ser 661, Ser 667, Ser 683) appear to be major sites phosphorylated both *in vitro* and *in vivo*. Chimeric peptide fragments in which mutant and wild-type linker peptides from PGP were linked to glutathione-S-transferase for purification on glutathione affinity columns were shown to be phosphorylated *in vitro* by PKC as expected from results using intact PGP. Mutant peptides lacking major sites of phosphorylation were poorly phosphorylated, as expected. A novel kinase has been purified to near homogeneity from KB-V1 plasma membranes which is a potent kinase for PGP *in vitro*. This kinase is not Ca-dependent, has a molecular weight of approximately 60,000, and is quite active in phosphorylating the GST-PGP peptides containing known phosphorylation sites. Of the three major phosphorylation sites on PGP, only two (Ser 661, Ser 667) are phosphorylated by the V1 kinase. The regulation and function of this novel kinase are not yet known, but its colocalization with PGP in the plasma membrane and its higher specific activity than PKC versus PGP and its peptides, suggest it may play a major role in determining the extent of

phosphorylation of PGP *in vivo*. To determine whether any of the five potential phosphorylation sites in the connecting region of PGP are essential for function, all five were replaced with Ala (to eliminate phosphorylation) or Asp (to preserve negative charge). Both constructs were inserted into pHaMDR expression vectors, and both conferred multidrug resistance indistinguishable in our initial analysis from wild-type PGP. The five Ala-PGP showed no evidence of phosphorylation of any other sites *in vitro* by PKC, PKA or VI kinase or *in vivo*. These results argue strongly that we have identified the major sites of phosphorylation of PGP, and that phosphorylation of these sites is not essential for function of PGP as a multidrug transporter, but do not rule out a role for phosphorylation in modulating an as yet unidentified function of PGP.

3. Molecular genetic studies on ATPase activity and substrate utilization: In addition to the biochemical approach described above, we have attempted to study the molecular requirements for ATPase activity by forming chimeras between PGP encoded by *MDR*1 and other members of the ATP binding cassette (ABC) superfamily of transporters. Initial studies show that both amino- and carboxy-terminal ATP sites from *MDR*2 (a related transporter expressed in liver which is essential for release of phosphatidyl-choline into bile) will substitute for the ATP sites in *MDR*1. However, ATP sites from CFTR do not form functional chimeras. Analysis of cell surface expression of chimeric PGPs indicates that the major reason for functional inactivation of chimeras is their failure to be expressed on the cell surface, either because they are improperly processed in the ER or Golgi, or degraded prior to reaching the cell surface. Using iodo-deoxyforskolin as an affinity label, the major substrate interaction sites in PGP have been localized to transmembrane domains (TMs) 5 and 6, TMs 11 and 12, and the extracellular and intracellular sequences associated with these TMs. A bicistronic expression vector (see below) using methotrexate resistance as a selectable marker, and *MDR*1 as the cDNA whose function is being analyzed, has been designed to allow introduction of random mutations into the substrate binding sites of *MDR*1 to define residues essential for substrate binding and specificity. The expression of the human *MDR*1 gene in *Saccharomyces cerevisiae* defective in the ERG6 gene (lack of ergosterol in the plasma membrane in ERG6 mutants sensitizes yeast to anti-cancer drugs and other cytotoxic agents which are MDR substrates) results in MDR. This heterologous expression system can be used to test the effect of various mutations in the ATP and substrate utilization sites.

4. *MDR*1 vectors for gene therapy: We have previously shown that expression of the human *MDR*1 gene in the bone marrow of transgenic mice under control of a chicken actin promoter results in resistance of bone marrow to the toxic effects of anti-cancer drugs. After several generations of inbreeding, the *MDR*1 transgenic mice no longer express detectable levels of PGP in their marrow. We have constructed three new lines of transgenic mice carrying the *MDR*1 gene under control of mouse and human immunoglobulin heavy chain upstream sequences. In one such line, human PGP mRNA is found in peripheral blood cells, and in bone marrow, spleen, thymus, and intestine, consistent with expression in lymphocytes. Studies are in progress to determine whether B lymphocytes expressing the human *MDR*1 cDNA are resistant to the cytotoxic effects of taxol and daunorubicin. Additional studies indicate that transduction of murine bone marrow with *MDR*1 retroviral vectors results in a selective advantage of the transduced cells over non-transduced cells in bone marrow. These observations have resulted in two new approaches to gene therapy which exploit our ability to select for cells expressing the human *MDR*1 cDNA. In the first approach, the *MDR*1 cDNA in a retroviral vector is used to confer MDR to bone marrow in patients undergoing intensive chemotherapy. We will be collaborating on two clinical trials (at NIH and at Columbia University Medical Center) to test this approach in breast cancer patients undergoing autologous bone marrow transplantation. The clinical trial at Columbia is part of a CRADA with GENETIX PHARMACEUTICALS CORPORATION (CACR-0150) initiated 11/08/93 and entitled "MDR Gene Expression in Hematopoietic Stem Cells." We have already developed one such vector in which both *MDR*1 and the Herpes simplex virus thymidine kinase (HSV-TK) gene are expressed in a single bicistronic expression vector. In this vector system, the *MDR*1 gene is under control of the retroviral LTR promoter, and the HSV-TK gene, on the same message, is independently

translated because it is downstream from an internal ribosome entry site (IRES). In this system, *MDR*1-expressing cells also express HSV-TK; this expression sensitizes recipient cells to ganciclovir and would allow the elimination of any cancer cells in the bone marrow which were inadvertently transduced by the *MDR*1 retroviral vector. The second approach involves similar bicistronic vectors in which *MDR*1 is the selectable gene, and the non-selectable gene may be any one of a variety of cDNAs encoding proteins defective in inborn errors of metabolism. To date, with the help and collaboration of Roscoe Brady, Edward Ginns and Harry Malech, we have constructed such vectors for glucocerebrosidase (defective in Gaucher disease), alpha-galactosidase (defective in Fabry disease), and the 91 kDa subunit of NADPH oxidase complex (defective in chronic granulomatous disease). All show expression of the non-selectable cDNA when cells are selected for *MDR*1 expression *in vitro*. Several successful approaches to transduction of murine bone marrow stem cells with *MDR*1 retroviral vectors are under development, including *ex vivo* selection and growth of stem cells in medium containing stem cell factor, IL-3, and IL-6, retroviral transduction of whole bone marrow or fetal hepatic hematopoietic elements, and liposome mediated transfer of retroviral DNA to bone marrow cells.

5. Mechanism of high level cisplatin resistance in cultured human KB adenocarcinoma and hepatoma cells: As detected on 2D gels, there are many alterations in expression of proteins from KB adenocarcinoma and BEL-7404 hepatoma cells selected for high level (approximately 100-fold) resistance to cisplatin. However, one prominent change seen in both KB and hepatoma cells is the overexpression of a protein of molecular weight 52-56 kDa. This protein has been isolated and microsequenced and found to be identical to the mitochondrial matrix heat shock protein hsp60. cDNA probes for hsp60 detect overexpression of mRNA in the highly cisplatin resistant cell lines. In collaborative studies with Dr. Thomas Hamilton (Fox Chase Cancer Center) and Shin-ichi Akiyama (Kagoshima University, Institute of Cancer Research), the highly resistant human cell lines developed in our laboratory have been shown to have decreased accumulation of cisplatin in cells and decreased platination of DNA. The relationship between decreased drug accumulation and increased expression of hsp60 is under investigation, but is as yet unexplained.

CRADA Information:

Contractor: GENETIX PHARMACEUTICALS CORPORATION
CRADA Number: CACR-0150
Date Contract Initiated: 11/08/93

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05599-04 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

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*)

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	K. Sakaguchi	Visiting Associate	LCB, NCI
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	S. J. Ullrich	Special Volunteer	LCB, NCI
	H. Sakamoto	Visiting Fellow	LCB, NCI

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LAB/BRANCH
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SECTION
Chemistry

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NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	0.0

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

B

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

Wild-type (wt) p53 induces the transcription of specific genes that are crucial for modulating cell cycle progression. Overexpression of wt-p53 has been shown to induce G1-arrest and to transactivate expression of the WAF1/CIP1 gene that encodes the p21 protein which is an inhibitor of cyclin-dependent kinases (CDK). Wt-p53-dependent G1-arrest is accompanied by decreased expression of the B-myb gene. We have shown that B-myb expression is required for cells to progress from G1 into S-phase and that high levels of ectopic B-*myb* expression rescues cells from p53-induced G1-arrest. This is the first evidence of a by-pass of the p53-induced WAF1/CIP1-mediated cell cycle regulatory pathway by a member of the myb oncogene family.

Human wt-p53 is a homotetramer in solution. The oligomerization domain has been mapped to the carboxyl-terminus. We have synthesized nine different peptides within this domain and shown that a peptide of 42 amino acids (residues 319-360) exhibits the strongest tetramerization and forms a unique antiparallel four helical bundle. The three-dimensional structure of the oligomerization domain has been solved by multi-dimensional magnetic resonance spectroscopy. The domain is a symmetric four helix bundle with adjacent helices oriented antiparallel and two antiparallel β sheets located on opposing faces of the molecule. This topology places a number of constraints on the location of the four DNA binding domains, one from each subunit. Further, this structure suggests a model whereby the C-terminal basic tail of wt-p53 can modulate the activity of the DNA binding domain.

Phosphorylation of Ser 392, the penultimate p53 residue, results in the activation of strong sequence-specific DNA binding. It was suggested that phosphorylation by casein kinase II may regulate DNA binding and the ability of p53 to function as a tumor suppressor. However, human p53 mutants that had Ser 392 changed to Ala or Asp were indistinguishable from wt-p53 in their ability to activate transcription and suppress RAS and adenovirus E1A mediated cell transformation. We conclude that phosphorylation of Ser 392 is not crucial for the ability of human p53 to activate specific expression *in vivo* or *in vitro*.

Major Findings:

The p53 tumor suppressor protein is a critical component of the G1 DNA damage checkpoint and transduction pathway and is important for maintaining genome stability. Growth arrest of mammalian cells at the G1/S boundary is induced when wt-p53 is artificially overexpressed. Treatment of mammalian cells with DNA damaging agents causes a transient prolongation of the half-life of p53. In contrast, there is no change in the level of p53 mRNA after treatment of cells with DNA damaging agents. These observations are consistent with the model that p53 may be relatively inactive in undamaged cells, but becomes activated in damaged cells as the result of post-translational modifications to p53 itself or to the system that is responsible for the normal rapid degradation of p53 in undamaged cells. This model of p53 activation is supported by the finding that unmodified mouse p53 produced in bacteria binds DNA relatively poorly, but binding is enhanced by phosphorylation at the penultimate serine residue or by proteins that bind near the carboxy-terminus of wt-p53. The function of p53 after DNA damage presumably is to temporarily arrest cell growth and allow time for repair to be made. In some cells overexpression of p53 induces apoptosis and induction of cell death in damaged cells may be another way by which p53 functions to reduce cell transformation. The G1 checkpoint mechanism is lost in human tumor cells through mutation of both p53 alleles or via expression of proteins that associate with p53 and abrogate its function. The p53 protein has been shown to be a modulator of transcription which can exhibit both positive and negative effects on gene expression. The functional biochemical versatility of p53 appears to depend on the cellular context, interactions with specific DNA sequences or other proteins and changes in p53 protein conformation.

Our studies have focused on elucidating the role that p53 plays in cell cycle regulation. We have shown that overexpression of wt-p53 in a human glioblastoma tumor cell line arrests cell cycle progression at or near a restriction point controlling the G1/S phase transition. Recently, a potential explanation for the molecular basis of G1 arrest induced in this model was provided by the demonstration that p53 transcriptionally activates the expression of the *WAF1/CIP1* gene which encodes a 21 kDa protein that is an inhibitor of G1 cyclin-dependent kinase activity. The interplay however, between growth-promoting genes and growth-suppressing genes in cell cycle control is poorly understood. We have reported that B-*myb* expression is dramatically decreased in G1-arrested cells. However, recent data demonstrate that constitutive high-level expression of *B-myb* in glioblastoma cells rescues cells from p53-induced G1-arrest mediated by the *WAF1/CIP1* gene. This observation raises the possibility that constitutive high-level expression of the B-myb transgene product may directly or indirectly subvert the repressing activity of nuclear factors regulating endogenous B-myb transcription. It has been suggested that B-myb promoter repression in Go could be mediated through the association of E2F with the retinoblastoma protein and/or related proteins. A simple model, therefore, might explain why endogenous *B-myb* mRNA levels are suppressed in glioblastoma cells. Induction of wt-p53 would transcriptionally activate *WAF1/CIP1* which would inhibit the derepressing activity of the G1/S cyclin-dependent kinases. Additional studies are being carried out to elucidate more fully the mechanism by which constitutive expression of B-myb rescues cells from wt-p53 induced arrest.

Recent studies clearly have indicated that the wt-p53 protein is a potent transcriptional activator, although the target genes of p53-mediated transactivation have not all been identified. The human glioblastoma cell line we have established (GM47.23), which contains hormone-inducible wt-p53 or mutant p53 transgenes, is ideally suited for studies aimed at identifying endogenous target genes of wt-p53 transactivation. A comprehensive directional library has been prepared from poly(A^+)-selected RNA transcripts. The screening of this library has been used with success, and the identification and cloning of a variety of cellular genes preferentially expressed in cells induced to overexpress wt-p53 protein is now in progress.

The study of wt-p53 has been hindered by its low levels of expression. However, both chemical synthesis of large peptide fragments or expression of these fragments in *E. coli* has provided a

means to analyze some of the structural characteristics of human p53. The carboxy-terminus of p53 is very basic and contains several functional elements: nuclear localization signals, an oligomerization domain and a non-specific DNA/RNA binding element. We have synthesized nine peptides from the carboxyl-terminal and have found that a peptide of 42 amino acids is the minimum size required for tetramer formation with a significant α-helix content. Extension in the N-terminal direction reduces tetramerization while extension in the C-terminal direction has little effect. The analysis of cross-linked tetramers was used to determine whether the monomers had a parallel or antiparallel orientation and these cross-linking studies suggest that the helices adopt an anti-parallel arrangement. Solution structural analysis by NMR of this unique four helix bundle has been completed. The tetramer is symmetric with the adjacent helices oriented antiparallel to each other and the non-adjacent opposing helices aligned in a parallel manner. The strands (residues 326-334) are arranged in two antiparallel β-sheets which lie on the outside of the helices and are located on opposite faces of the molecule. This topology allows the DNA binding domains of p53 (residues 102-292) to be in close proximity; the C-terminal basic tail may, therefore, interfere with specific complex formation and modulate the activity of the DNA-binding domain.

p53 is phosphorylated *in vivo* at several sites including three in the amino-terminal transactivation region (Ser 9, 15 and 33), one just before the major nuclear localization signal (Ser 315), and one at the carboxy terminus (Ser 392). Three of the five identified phosphorylation sites in human p53, Ser 15, 315 and 392, are located in sequences that are well conserved between species. Ser 392 can be phosphorylated by casein kinase II (CKII) *in vitro*. Phosphorylation by CKII was reported to enhance sequence specific DNA-binding by bacterially-expressed p53. Mutation of Ser 386 codon of mouse p53 was also reported to abolish p53's ability to suppress growth. To determine if Ser 392 of human p53 is essential for p53 mediated cell growth control, we constructed mutants that changed Ser 392 to alanine or aspartic acid and constructed cell lines that expressed these p53 mutants in response to dexamethasone. When overexpressed in stably-transformed derivatives, p53-Ala^{392} was as effective as wt-p53 in the ability to arrest cell growth. Furthermore, p53-Ala^{392} and p53-Asp^{392} activate transcription of a construct with multiple copies of a p53 responsive element linked to a luciferase reporter gene and suppressed foci-formation by activated *RAS* and adenovirus12 *E1A* oncogenes as well as wt-human p53. Thus, a role for Ser 392 phosphorylation if any, is still unclear.

The amino-terminal transactivation domain of p53 is phosphorylated at several sites by several different kinases, including a casein kinase I-like activity, the DNA-activated protein kinase, the UV-activated cytoplasmic protein kinase and the mitogen activated protein kinase, MAPK. The amino-terminal transactivation domain is involved in p53 interactions with several host and viral proteins. Thus, phosphorylation of p53 at the N-terminal residues is likely to affect the interaction of p53 with these proteins. We have constructed mutants with Ser 15 changed to Ala. This site is phosphorylated *in vitro* by DNA-activated protein kinase, DNA-PK.

When expressed in stably transformed derivatives, p53-Ala^{15} was slightly less efficient than wt-p53 in arresting cell growth, but the half-life of the mutant was greatly enhanced. These findings are consistent with a role for phosphorylation of Ser 15 by DNA-PK in regulating p53 stability. DNA-PK is activated when it binds to the ends of DNA fragments or to gaps in closed circular DNAs. These are structures that can be generated by DNA-damaging agents or may be transiently created as a consequence of the action of repair enzymes. It is not unlikely therefore, that two or more of these kinases function synergistically to produce the rapid changes in p53 activity that would be required to detect a DNA damage signal.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08715-16 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

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
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Others:	J. Reed	Research Associate	LCB, NCI
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

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

B

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

Procathepsin L is the precursor to the lysosomal cysteine protease cathepsin L. It is secreted in large amounts by malignantly transformed mouse cells, and by cells treated with tumor promoters or growth factors. The function of procathepsin L in malignancy is not known, but involvement in tumor invasion and metastasis, and in suppresion of the immune response to tumors has been suggested. In normal tissues, procathepsin L is secreted by the liver, osteoclasts, and Sertoli cells, indicating possible involvement in bone resorption and sperm maturation. To study the biological function of procathepsin L, and the related lysosomal cysteine protease cathepsin B, which is secreted at somewhat lower levels by malignant cells, we have attempted to inactivate these cathepsin genes by insertional mutagenesis in cultured B16 melanoma cells (a mouse melanoma line which forms lung metastases which can be easily quantitated), and mouse embryonic stem (ES) cells. "Knockout" vectors carrying two Herpes simplex virus thymidine kinase ("suicide") genes and containing genomic DNA encoding cathepsins L and B have been prepared in which DNA from B16 melanoma cells or ES cells (cathepsin L DNA) was insertionally inactivated with a bacterial neomycin-resistance cDNA. Insertion of this vector by homologous recombination after electroporation should result in G418 and ganciclovir resistance of recipient cells. For cathepsin B, attempts to inactivate the respective endogenous genes in B16 melanoma cells were unsuccessful, suggesting either that inactivation of a single copy of cathepsin B from these cells is a lethal event, or that B16 melanoma cells are poor candidates for insertional mutagenesis owing either to an inadequate recombination apparatus, or a hostile environment for incoming DNA. Attempts to inactivate the cathepsin L gene in B16 cells and in ES cells are in progress in the hope of generating a transgenic mouse lacking this lysosomal protease to test hypotheses about cathepsin L function.

Major Findings:

1. Malignant transformation of mouse cells has been observed to be associated with large increases in secretion of procathepsin L, the precursor to the lysosomal cysteine protease cathepsin L. Tumor promoters and growth factors have been shown to upregulate the expression of cathepsin L. Other evidence from our laboratory, which supports a role for the involvement of cathepsin L and its precursor in the malignant process, comes from studies which show increased levels of cathepsin L mRNA expression in various human cancers. The exact way in which cathepsin L is involved in malignancy is unknown, but many studies suggest that cathepsin L may be involved in tumor metastasis and immune suppression. In addition to cathepsin L's involvement in cancer, studies from normal tissues show that procathepsin L is secreted by the liver, osteoclasts, and Sertoli cells suggesting possible involvement in bone resorption and sperm maturation. To better understand how cathepsin L and the related lysosomal cysteine protease, cathepsin B (which is secreted at lower levels in malignant cells compared to cathepsin L) may be involved in these biologic processes, we have tried to inactivate these cathepsin genes by insertional mutagenesis in cultured B16 melanoma cells and embryonic stem (ES) cells. To enhance our ability to achieve a homologous recombination (gene knockout) event, we have isolated isogenic (same cell line) DNA fragments from appropriate genomic libraries and cloned them into a knockout vector carrying two Herpes simplex virus thymidine kinase ("suicide") genes and a bacterial neomycin resistance cDNA which insertionally inactivates the coding region of the cathepsin genes. Homologous recombination after transfection by electroporation should result in recipient cells carrying G418 (neomycin analog) resistance and ganciclovir resistance.

2. Attempts to inactivate the cathepsin B gene in B16F10 cells capable of metastasis to lung have been unsuccessful. We used a targeting vector with 5.2 kb of cathepsin B genomic DNA. Of 400 G418-resistant, ganciclovir resistant clones, none showed evidence of homologous recombination using either Southern blots or a PCR-based assay. We attribute this failure to obtain homologous recombinants to a low frequency of homologous recombinant in B16 cells, or to lethality of the cathepsin B knock-out event. Similar efforts for cathepsin L in both B16 cells and ES cells have been initiated.

Publications:

Gottesman MM. Cathepsin L and cancer. In: Bond JS, Barrett AJ, eds. Proceedings of the 9th ICOP Conference: Proteolysis and Protein Turnover; ch. 36. London: Portland, 1993;247-51.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 09100-09 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

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

PI:	V. Hearing	Research Biologist	LCB, NCI

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.7	2.0	2.7

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

B

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

This project is aimed at characterizing parameters important to the growth and differentiation of melanocytes and their significance to critical properties of transformed melanocytes (termed malignant melanoma) such 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 pigmentation-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 regulatory catalytic controls can differ dramatically, and their effects on the functional and photoprotective properties of melanins are being actively studied. Our laboratory has continued to characterize melanoma-specific antigens abnormally expressed by transformed melanocytes which play a role in the host's immune responses to tumor growth. Monoclonal antibodies to one of those antigens, termed B700, specifically cross-react with human melanoma and have proven useful as highly specific probes for detecting malignant melanoma. Intravenous treatment of tumor-bearing hosts with those antibodies provides significant protection against metastatic growth. B700 is the immunodominant antigen in a melanoma vaccine being developed that has proven to be efficacious for host survival in a novel model for spontaneous melanoma metastasis.

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Major Findings:

1) Melanogenesis. Our studies have investigated the regulatory mechanisms involved in the basal differentiation of mammalian melanocytes and in their ability to respond to such stimuli of differentiation as ultraviolet (UV) light or melanocyte stimulating hormone (MSH). We have now generated a panel of specific peptide antibodies which recognize distinct melanogenic proteins; those antibodies have been used to purify the corresponding proteins by immune-affinity chromatography and to subsequently characterize their structural and catalytic melanogenic function(s). In combination with specific nucleotide probes for those genes, we have been able to characterize the sequence of events involved in the response mechanisms of melanogenic stimulation at the transcriptional, translational and post-translational levels. We have found that although several of those genes are closely related (belonging to a tyrosinase gene family) and that all are specifically expressed only by melanocytes, they are independently regulated at the transcriptional level following stimulation of differentiation. The genes involved are encoded at the following murine loci: *albino* encodes tyrosinase, a trifunctional catalytic enzyme essential for melanogenesis; *brown* encodes TRP1, which functions uniquely as DHICA oxidase; *slaty* encodes TRP2, which has a specific function as DOPAchrome tautomerase; *silver* encodes Pmel17, which functions as a melanosomal matrix protein with an as yet undetermined catalytic action; and *pink* encodes P protein, thought to function as a melanosomal tyrosine transporter (tyrosine being the critical melanogenic substrate). All five of these genes have human homologues which have also now been cloned and shown to be similarly specifically expressed by human melanocytes. Mutations at three of those loci (*albino*, *brown* and *pink*) elicit various types of human albinism, termed OCA1, OCA2 and OCA3. We have now characterized the specific lesions elicited by many of those human albino mutations and have characterized the catalytic function that is disrupted in the mutant protein; the distinct effects of some of those mutations on tyrosine hydroxylase function, but not the DOPA oxidase or DHI oxidase functions, suggests that they occur at distinct catalytic sites. Other mutations affect all catalytic functions and/or the thermostability of the mutant protein; all mutations have the end result of severe hypopigmentation associated with developmental abnormalities. We have now set up assays to

measure ligand and cofactor binding to mutant proteins, translational rates and intracellular stability of the mutant proteins, and that study will eventually characterize the more than 40 distinct mutations that have been defined in tyrosinase-negative albino patients. Comparable studies are being initiated to study the tyrosinase-positive albinism caused by mutations at the *brown* and *pink* loci. The stabilizing interactions of these melanosomal-localized gene products have also been characterized, and many of the mutations involved at those gene loci seem to affect the stability of this melanogenic complex and its resistance to degradation in the melanosome *in vivo*. We have continued our studies aimed at characterizing a low molecular weight inhibitor of pigment production; this specific inhibitor plays an important post-translational 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 have continued our characterization of the structure and function of the melanins produced by mammalian melanocytes 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, which will be 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 KAO CORPORATION since April 22, 1993; that project has begun 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 by the host have continued to emphasize the importance of the B700 antigen in host immune mechanisms. 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 the murine B700 and the human M66 antigen are immunologically cross-reactive, though not identical. We have shown that, following vaccine therapy with B700 or with melanoma-derived extracellular antigens, melanoma bearing mice produce specific complement-dependent cytotoxic antibodies which have the same specificity elicited by immunization with irradiated cells (or cell extracts). There are also significantly enhanced cell mediated immune responses (including natural killer cell and cytotoxic T cell activities) in vaccinated mice. The sum of these immune responses leads to a dramatic increase in the host survival, which can be even further prolonged with concomitant interleukin-2 therapy. B700 is the immunodominant antigen eliciting those beneficial antitumor effects. We have successfully developed a murine monoclonal antibody, termed H-2-3-3, 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 tissues, including skin. In view of the specificity of this antibody to melanoma cells, and the fact that it can provide partial protection to mice against metastatic spread of the tumor, future studies will assess its potential use following conjugation to various toxins. We have been studying 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 level of any protease required for metastasis is being addressed using gene knockout technology.

Crada Information:

Contractor: KAO CORPORATION

CRADA Number: CACR-0159
Date Contract Initiated: 04/22/93

Publications:

Aroca P, Urabe K, Kobayashi T, Tsukamoto K, Hearing VJ. Melanin biosynthesis patterns following hormonal stimulation, J Biol Chem 1993;268:25650-5.

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Palumbo A, Napolitano A, DeMartino L, Vieira WD, Hearing VJ. Specific incorporation of 2-thiouracil into biological melanins, Biochim Biophys Acta 1994;in press.

Shrayer D, Bogaars H, Gersten DM, Hearing VJ, Maizel A, Wanebo H. A nude mouse model to study passive humoral immunotherapy directed to the B16 F10 murine melanoma, J Surg Oncol 1994;in press.

Kobayashi T, Urabe K, Winder AJ, Jiménez-Cervantes C, Imokawa G, Brewington T, Solano F, García-Borrón JC, Hearing VJ. Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis, EMBO J 1994;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 09101-02 LCBGY

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Characterization of NCp7 and Viral Peptides Bound to MHC Class I of HIV-1 Virions

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

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	N. Zambrano	Visiting Fellow	LCB, NCI
	M. Lewis	Senior Scientist	BEIP, NCRR
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E. T. Baldwin, J. W. Erickson, FCRDC, NCI

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Chemistry

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

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

B

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

The title of the project has been renamed. Last year's title was Analysis of Complexes Between Zinc Finger Proteins and DNA or RNA of HIV-1 Virion.

The nucleocapsid protein NCp7 of HIV-1 is important for encapsidation of the viral genome, RNA dimerization, and primer tRNA annealing *in vitro*. We have shown that a region in the 5'-terminal of the HIV-1 genomic RNA can be folded into two stem-loop structures which are recognized by NCp7. We have performed analytic ultracentrifugation and chemical interference experiments using a chemically synthesized NCp7 (1-55) peptide and 19 and 44-nucleotide RNAs. The results revealed that one molecule of NCp7 peptide forms a complex with two molecules of either type of RNA containing the stem-loop structures. Nucleotides critical for NCp7 binding have been mapped to a single site located within the first loop. Since the 19 nucleotide RNA retained the ability to form the 2:1 RNA to peptide complex, it appears that this oligonucleotide represents the minimal NCp7 binding site for HIV-1 RNA dimerization. The analysis of the complex between the 19-nucleotide RNA and the NCp7 by NMR and X-ray crystallography will clarify the mode of action of these two components and aid in the design of antiviral agents capable of inhibiting HIV-1 NCp7 functions. The principal neutralizing determinant of HIV-1 is an intrachain disulphide-bridged loop (V3) in the third hypervariable region of gp120. Peptide sequences from the V3 loop of HIV-1 strain MN were engineered into N-terminal region of the major coat protein of the filamentous bacteriophage fd. These constructs evoke high titers of antibodies in mice without adjuvants, which cross-react with other strains of HIV-1 and are capable of neutralizing the virus. The bacteriophage display offers a powerful means of designing inexpensive vaccines against HIV-1. Virus-specific cytotoxic T lymphocytes (CTL) may be an important host defense mechanism in the control of HIV-1 replication. CTLs that recognize peptide fragments of various HIV-1 proteins have been previously characterized, and in some cases the optimally active synthetic peptides from HIV-1 infected cells have been defined. We have initiated an analysis of the naturally processed peptides from HIV-1 infected cells independent of their recognition by HIV-specific CTL. This approach should identify both viral and cellular peptides capable of eliciting cellular cytotoxic responses and prove valuable in the development of a vaccine.

Major Findings:

1) Characterization of a fragment of HIV-1 nucleocapsid protein p7 with a high affinity RNA-binding site by computerized structure prediction analysis, chemical interference, and equilibrium analytical ultracentrifugation studies.

The nucleocapsid protein NCp7 of HIV-1 is able to promote dimerization of viral genomic RNA and is essential for virus RNA encapsidation. In a recent paper, we have shown that RNA sequences necessary to promote RNA dimerization in the presence of NCp7 protein can be folded in a stem and loop structure. Gel mobility shift analyses have indicated that the NCp7 forms two complexes with a 44 nucleotide HIV-1 RNA segment (positions 281-324) containing the dimerization region. However, we were unable to determine the stoichiometry of components in complex formation from the gel shift experiments. We further analyzed the complex structures by analytical ultracentrifugation. Contrary to the observation in gel shift experiments we found a single complex by analytical ultracentrifugation with a stoichiometry of one peptide to two RNAs.

In order to define the minimal size of RNA which would still react with NCp7, a 19 nucleotide RNA which included stem and loop 1 was studied. This RNA also formed a complex with only one peptide and two RNAs. NCp7 contains two zinc finger domains. If these are the binding sites for stem-loop 1, we can expect two binding sites for the RNAs. The measured binding affinities for the first site are essentially identical; the binding to the second site is significantly weaker. The possible causes for this lower affinity exhibited by the second binding site are not clear at this time. We have also attempted to define specific nucleotides within the 44-nucleotide RNA required for NCp7 binding. These experiments identified four nucleotides clustered in Loop I which, when modified, strongly interfered with NCp7 binding. This binding site lies in a region highly conserved among different HIV-1 isolates and includes the major 5' splice junction. The identification of this site provides an opportunity to analyze the complex of the 19-nucleotide RNA with the NCp7 by NMR and X-ray crystallography and determines the mode of interaction of these two components. Such studies will aid in the design of antiviral agents capable of inhibiting HIV-1 NCp7 functions and genomic RNA packaging.

2) Analysis of the immunogenicity of peptide epitopes of HIV-1 gp120 on filamentous bacteriophage and identification of MHC-bound HIV-1 peptides expressed by chronically infected cell lines.

The genome of bacteriophage fd has been engineered to allow foreign amino acid sequences to be incorporated into exposed regions of the major and one of the minor coat proteins of the intact virion. This has become the basis of a new technology, phage display. Peptides displayed in this way evoke strong and highly specific immune responses in various animals. A major feature of the 36-residue V3 loop sequence of HIV-1 gp120 is a conserved GPGR motif, flanked by residues that differ between various HIV-1 isolates. We selected the sequence IHIGPGRAYTT of HIV-1 MN isolate for display into the major coat protein (gVIIIp). From SDS-polyacrylamide gel electrophoresis we found that the coat protein contained approximately 900 MN gp120 determinants displayed on each hybrid virion. Inoculation of mice with hybrid MN phage elicited a strong antibody response in the absence of any adjuvant. The sera also cross-reacted with gp120 from HIV-1 IIIB infected cells and neutralized not only HIV-1 MN but the IIIB isolate as well. These results indicate that the MN peptide insert mimics the natural epitope and the antibodies generated exhibit a high specificity for the native protein. This approach, therefore, has potential for exploring the immune response to defined sequence epitopes.

In most viral infections, cellular immunity plays a significant role for the control of a persistent infection. In the case of HIV-1 infection, little information is known about the biological role of the CTL in the course of this long infectious process. Numerous reports have described ENV, GAG, POL and NEF-specific CTL epitopes in HIV-1-infected individuals, but to date there is no information on the

efficiency of the control of virus replication related to the specificity of the CTL activity in humans. We have taken a different approach and established long-term cultures to study cell lines chronically infected with HIV-1. To analyze the HIV-1-derived peptides which are naturally processed and presented by Class I molecules, a B cell line and a T-cell line are currently being used. MHC-bound peptides from chronically infected T-cell and B-cell lines were extracted from 10^9 cells and fractionated by RP-HPLC. Infected and uninfected cell peptide isolates were simultaneously compared. Interestingly, we have found that in chronically infected cells very low levels of HIV-1 specific peptides are present. Peptides derived from cellular components in chronically infected cells are increased relative to the uninfected cells. This finding suggests that HIV-1 can modulate the level of peptide presentation or the specificity for presentation of non-self antigens during the course of chronic infection. This analysis is currently under way and could have significant implications for a 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 type1 nucleocapsid protein, Proc Natl Acad Sci USA 1993;90:5219-23.

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SUMMARY STATEMENT

ANNUAL REPORT
LABORATORY OF CELLULAR ONCOLOGY
DCBDC, NCI
October 1, 1993 through September 30, 1994

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:

Analysis of Papillomaviruses

Papillomaviruses (PVs) infect the epithelia of animals and man where they generally induce benign proliferation at the site of infection. However, there is a strong association between malignant progression of human genital lesions and certain HPV types, most frequently HPV16.

We have used recombinant baculoviruses to produce self-assembled virus-like particles (VLP) from several human and animal PV types via baculovirus vectors. The VLPs are properly folded and induce high tier neutralizing antibodies, which are directed against conformational epitopes. Vaccines based on CRPV VLPs were able to prevent experimental CRPV infection in rabbits. We have identify HPV16 L1 clones from primary lesions that, unlike the prototype L1 used in previous studies, efficiently assembles into particles. Rabbit antibodies to the efficiently assembled HPV16 L1, but not the prototype L1, blocked the binding of HPV16 VLPs to human keratinocytes. These types of particles might be considered as a candidate for a prophylactic vaccine to prevent HPV16 infection.

Using the HPV16 VLPs, we have developed an ELISA that detects serum antibodies in the majority of women positive for HPV16 by DNA-based methods. As with cervical cancer risk, seropositivity strongly correlates with persistent HPV16 infection. We have also demonstrated an association between the risk of cervical cancer and the prevalence of VLP ELISA positivity for several populations.

We have analyzed the cellular effects of E2F-1, a transcription factor that is released from pRb when it binds to HPV16 E7, which is selectively retained and expressed in genital HPV associated cancers. An E2F-1 cDNA was toxic to keratinocytes and fibroblasts when expressed as high levels and this toxicity required the DNA binding but not trans-activating function of E2F-1. At lower levels, E2F-1 could functionally substitute for a pRB binding defective mutant of E7 but not for E7 entirely in the keratinocyte immortalization assay. Therefore, it appears that limited activation of E2F-1 is an important, but not the sole, function of E7.

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. The inhibition was at the level of 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 GRF, a gene 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 GRF 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.

Role of protein kinases in modulating cell growth and malignant transformation

The focus of this project is to better elucidate the possible involvement of trans-membrane signal transmission systems in the regulation of cell growth, in malignant transformation, and in cellular resistance to chemotherapeutic drugs. Previously, it was shown that phosphate ion (Pi) uptake into NIH 3T3 cells was stimulated with activation of protein kinase C (PKC), and that different isotypes of PKC may act to increase sodium-dependent phosphate uptake, but by different mechanisms. Our results now indicate that the gibbon ape leukemia virus (GaLV) cell surface receptor (GLVR1) can function as a sodium-dependent phosphate transporter. Overexpression of GLVR1 in NIH 3T3 cells increased Pi uptake, while infection of cells overexpressing GLVR1 with virus (GaLV) blocks receptor-dependent ion transport. These results suggest that GLVR1 is a ubiquitously expressed, high affinity phosphate transporter which presumably functions to maintain cellular phosphate levels.

Multidrug resistant MCF-7/MDR cells exhibit elevated levels of a modified form of PKC alpha at the nucleus. The nuclear levels of PKCa were found to selectively increase with increasing degrees of drug resistance. These results suggest that elevated levels of PKC alpha at the nucleus may play a role in modulating nuclear events to promote the onset or maintenance of multidrug resistance in MCF-7 cells.

A new epitope-tagging adapter sequence was designed which can be inserted into vectors of choice to faciliate the expression and epitope-tagging of any polymerase chain reaction (PCR)-generated cDNA fragment into either bacterial or mammalian cells. The epitope-tagged recombinant proteins can be detected, isolated, and characterized with a specific anti epitope tag antibody. This epitope-tagging construct was used in experiments to identify the domain responsible for the serum- and PMA-induced gel mobility shift noted with Raf protein kinase. Results indicate that modification(s) within the 33 kDa C-terminal fragment of Raf may be responsible for the observed shift in gel mobility.

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

PROJECT NUMBER
Z01 CR 03663-18 LCO

PERIOD COVERED
October 1, 1993 through September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Role of protein kinases in modulating cell growth and malignant transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, Laboratory, Branch, NCI)

PI:	W. B. Anderson	Research Chemist	LCO, NCI
Other:	Z. Olah	Visiting Associate	LCO, NCI
	C. Lehel	Visiting Fellow	LCO, NCI
	A. Ferrier	IRTA Fellow	LCO, NCI
	G. Petrovics	Visiting Fellow	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:
4.5	4.5	0

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 focus of this project is to better elucidate the possible involvement of trans-membrane signal transmission systems in the regulation of cell growth, in malignant transformation, and in cellular resistance to chemotherapeutic drugs. Previously, it was shown that phosphate ion (Pi) uptake into NIH 3T3 cells was stimulated with activation of protein kinase C (PKC), and that different isotypes of PKC may act to increase sodium-dependent phosphate uptake, but by different mechanisms. Our results now indicate that the gibbon ape leukemia virus (GaLV) cell surface receptor (GLVR1) can function as a sodium-dependent phosphate transporter. Overexpression of GLVR1 in NIH 3T3 cells increased Pi uptake, while infection of cells overexpressing GLVR1 with virus (GaLV) blocks receptor-dependent ion transport. These results suggest that GLVR1 is a ubiquitously expressed, high affinity phosphate transporter which presumably functions to maintain cellular phosphate levels.

Multidrug resistant MCF-7/MDR cells exhibit elevated levels of a modified form of PKC alpha at the nucleus. The nuclear levels of PKCa were found to selectively increase with increasing degrees of drug resistance. These results suggest that elevated levels of PKC alpha at the nucleus may play a role in modulating nuclear events to promote the onset or maintenance of multidrug resistance in MCF-7 cells.

A new epitope-tagging adapter sequence was designed which can be inserted into vectors of choice to faciliate the expression and epitope-tagging of any polymerase chain reaction (PCR)-generated cDNA fragment into either bacterial or mammalian cells. The epitope-tagged recombinant proteins can be detected, isolated, and characterized with a specific anti epitope tag antibody. This epitope-tagging construct was used in experiments to identify the domain responsible for the serum- and PMA-induced gel mobility shift noted with Raf protein kinase. Results indicate that modification(s) within the 33 kDa C-terminal fragment of Raf may be responsible for the observed shift in gel mobility.

Cooperating Units:

Lab. of Cellular Biology, NIMH, NIH, Drs. M. V. Eiden and C. A. Wilson
Lab. of Carcinogenesis and Tumor Promotion, NCI, NIH, Dr. P.M. Blumberg
Clin. Neuroscience Branch, NINDS, NIH, Dr. G. Jakob
Hormel Inst. , Univ. of Minnesota, Austin, MN., Dr. Z. Kiss
Lab. Physiopath. Development, Paris, France, Drs. D. Evain-Brion and F. Raynaud

Major Findings:

1. Protein Kinase C. Protein kinase C (PKC) is a phospholipid-dependent serine threonine protein kinase of fundamental importance in transmembrane signal transmisson. Thus, it is of importance to better characterize the regulatory properties of PKC, and to better define its possible role in cell growth regulation, tumor promotion, and resistance to drugs of the natural products class. Sodium-dependent phosphate (Pi) transport has been shown to be under homonal regulation, and we have shown that activation of PKC in turn stimulates Na/Pi-uptake. Other studies established that different PKC isotypes may contribute to the regulation of Pi transport, but through distinct mechanisms. Now we have observed that a cell surface virus receptor may be a Pi transporter. The primate type C retrovirus gibbon ape leukemia virus (GaLV) uses a widely expressed, multiple membrane-spanning protein as its cell surface receptor (GLVR1) on human cells. We have found that GLVR1 can function as a high-affinity sodium-dependent phosphate transporter. Expression of GLVR1 cDNA in murine cells was shown to result in a marked increase in sodium-dependent Pi transport, and GLVR1 was found to have a 3-4 fold higher affinity for phosphate than other mammalian phosphate transporters. Further, infection of GLVR1-expressing cells by GaLV resulted in the complete blockage of GLVR1-specific Pi uptake. These results suggest that GLVR1 is a Na-dependent Pi transporter which differs from other mammalian phosphate transporters in structure, affinity for phosphate, and function.

Several lines of evidence have indicated the possible involvement of PKC in modulating cellular resistance to antitumor drugs. PKC activities are increased in multidrug-resistant human breast cancer MCF-7/MDR cells compared to control, drug-sensitive MCF-7/WT cells. We also have shown a significant increase in the levels of the PKC alpha isotype in the nuclear fraction of MCF-7/MDR cells. Studies now have been carried out with a series of multidrug-resistant MCF-7 cells with increasing degrees of drug resistance. Immunoblot analysis of proteins extracted from nuclear, cytosolic, and plasma membrane fractions demonstrated that the levels of nuclear PKC alpha increased as the degree of drug resistance increased. These results indicate a good correlation between the level of nuclear PKC activity and the degree of drug resistance in these MCF-7 human breast cancer cells. Since PKC is regulated by diacylglycerol generated in response to stimulation of various phospholipase activities, changes in phospholipase activities also might be expected in MDR cells. Thus, the phospholipase D (PLD)-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) were examined in drug-sensitive and multidrug-resistant MCF-7 cells. In MCF-7/WT cells, treatment with phorbol ester (PMA) did not alter the hydrolyisis of either phospholipid. However, PMA treatment of MCF-7/MDR cells greatly (29-fold) increased the hydrolysis of PtdEtn, but had no effect on the hydrolysis of PtdCho. In addition, sphingosine and hydrogen peroxide also significantly stimulated PtdEtn hydrolysis in MCF-7/MDR cells. These results indicate that the PLD-mediated hydrolysis of PtdEtn is differentially and selectively regulated by PKC activators in MCF-7/MDR cells.

2. Raf protein kinase. Another cytosolic protein kinase implicated in intracellular signal transduction is the Raf-1 protein kinase (Raf-PK), encoded by a member of the raf proto-oncogene family. We were hindered in studies of Raf-PK by the lack of molecular probes specific for the truncated fragments and domains of this enzyme. To facilitate the study of Raf-PK a new gene-tagging insert sequence was designed to conveniently introduce epitope-tagged polypeptides into bacteria and mammalian cells. The tagging insert encodes a peptide derived from the last 12 amino acids of PKC epsilon to serve as a C-terminal epitope tag of the expressed protein. This epitope-tagging adapter

sequence was designed so that it can be readily inserted into vectors of choice to facilitate the cloning of any polymerase chain reaction (PCR)-generated cDNA fragment into either bacterial or mammalian cells. The expressed epitope tagged proteins can be readily detected with antibody specific for the epitope peptide. To characterize the regulatory properties and subcellular distribution of holo Raf-PK , as well as 3 fragments covering the entire length of Raf-1-PK, we used the epitope-tagging vector construct to overproduce epitope-tagged holo Raf and the Raf fragments in NIH 3T3 cells. Experiments were carried out to identify the Raf-PK domain responsible for the upward shift in gel mobility noted with serum and PMA treatment of NIH 3T3 cells. The overexpressed, epitope tagged holo Raf-PK and also the epitope-tagged 33 kDa C-terminal fragment of Raf exhibited a serum-and PMA-induced shift in gel mobility, while the 35 kDa N-terminal fragment did not show a change in gel mobility under these conditions. These results suggest that modification(s) within the 33kDa C-terminal portion of Raf may be responsible for the band shift observed with serum and PMA treatment of serum-deprived NIH 3T3 cells.

Publications:

Lehel, C., Olah, Z., Mischak, H., Mushinski, J.F., and Anderson, W.B. Overexpressed protein kinase C-delta and-epsilon subtypes in NIH 3T3 cells exhibit subcellular localization and differential regulation of sodium-dependent phosphate uptake. J. Biol. Chem. 1994; 269: 4761-4766.

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

PROJECT NUMBER: Z01 CB 08905-13 LCO

PERIOD COVERED: October 1, 1993 through September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Tumor gene expression in vitro and in vivo

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, Laboratory, Branch, Institute)

PI:	D. R. Lowy	Chief, LCO	LCO, NCI
Other:	J. E. DeClue	IRTA Fellow	LCO, NCI
	M. Johnson	IRTA Fellow	LCO, NCI
	A. Konig	Special Volunteer	LCO, NCI
	S. Felzmann	General Fellow	LCO, NCI
	A. G. Papageorge	Microbiologist	LCO, NCI
	W. C. Vass	Biologist	LCO, NCI

COOPERATING UNITS (if any)

see next page

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.7	5.7	2.0

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.)

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. The inhibition was at the level of 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 GRF, a gene 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 GRF 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.

PHS 6040 (Rev. 5/92)

Cooperating Units:

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 Medicione, Dr. R. White

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 GRF. 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 Ras target. 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. GRF is a Ras-specific guanine nucleotide exchange factor that activates Ras.

Schwannoma cell lines from patients with neurofibromatosis have 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 Ras-specific GTPase stimulation negatively regulates Ras. We 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. Their growth inhibition was demonstrated to be at the level of Ras, since cells overexpressing neurofibromin displayed much lower transformating activity by mutantationally activated ras genes whose encoded proteins were resistant to the GTPase accelerating activity of neurofibromin, while the same cells had a normal response to transformation by mutationally activated a raf gene, whose encoded product lies just downstream of Ras. Furthermore, in melanoma cell lines, 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. These results demonstrate that NF1 can induce differentiation. It remains to be determined if this activity dependent in independent of Ras.

We recently identified four types (I-IV) of apparently full-length cDNAs from GRF. 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 GRF 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 GRF 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) and 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:

Lowy, D.R., and Willumsen, B.M. Function and regulation of Ras. Ann. Rev. Biochem. 1993;62:851-91.

Lowy, D.R., Johnson, M.R., DeClue, J.E., Cen, H., Zhang, K., Papageorge, A.G., Vass, W.C., Willumsen, B.M., Valentine, M.B., and Look, T.A. Cell transformation by *ras* and regulation of its protein product. In CIBA Symposium 176, The GTPase Superfamily, John Wiley & Sons, New York, 1993, pp. 67-80.

Lowy, D.R. Carcinogenesis: Viral. In Dermatology in General Medicine, edited by Fitzpatrick, T.B., et al., fourth edition, McGraw-Hill, New York, 1993, pp. 793-796.

Lowy, D.R. Viral Diseases: General considerations. In Dermatology in General Medicine, edited by Fitzpatrick, T.B., et al., fourth edition, McGraw-Hill, New York, 1993, pp. 2507-2512.

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DeClue, J.E., Vass, W.C., Johnson, M.R., Stacey, D.W., and Lowy, D.R. Functional role of GTPase-activating protein in cell transformation by pp60v-src. Mol. Cell. Biol. 1993;13:6799-6809.

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DeClue, J.E., and Lowy, D.R. Molecular aspects of oncogenesis. In The Molecular Basis of Blood Diseases, edited by Stamatoyannopoulos, G., et al., second edition, W. B. Saunders Company, Philadelphia, 1994, pp. 789-833.

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

PROJECT NUMBER
Z01 CB 09052-06 LCO

PERIOD COVERED
October 1, 1993 through September 30, 1994

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

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

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	H. Chenglong	Visiting Fellow	LCO, NCI
	M. M. Okun	Medical Staff Fellow	LCO, NCI
	N. L. Hubbert	Microbiologist	LCO, NCI
	J. V. Taub	Bio Lab Technician	LCO, NCI

COOPERATING UNITS (if any)
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Papillomavirus Unit, Pasteur Institute, Paris, France, Drs. G. Orth and F. Breitburd
Dematology Department, University of Vienna, Austria, Dr. R. Kimbauer

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.0	5.0	2.0

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.)

Papillomaviruses (PVs) infect the epithelia of animals and man where they generally induce benign proliferation at the site of infection. However, there is a strong association between malignant progression of human genital lesions and certain HPV types, most frequently HPV16.

We have used recombinant baculoviruses to produce self-assembled virus-like particles (VLP) from several human and animal PV types via baculovirus vectors. The VLPs are properly folded and induce high tier neutralizing antibodies, which are directed against conformational epitopes. Vaccines based on CRPV VLPs were able to prevent experimental CRPV infection in rabbits. We have identify HPV16 L1 clones from primary lesions that, unlike the prototype L1 used in previous studies, efficiently assembles into particles. Rabbit antibodies to the efficiently assembled HPV16 L1, but not the prototype L1, blocked the binding of HPV16 VLPs to human keratinocytes. These types of particles might be considered as a candidate for a prophylactic vaccine to prevent HPV16 infection.

Using the HPV16 VLPs, we have developed an ELISA that detects serum antibodies in the majority of women positive for HPV16 by DNA-based methods. As with cervical cancer risk, seropositivity strongly correlates with persistent HPV16 infection. We have also demonstrated an association between the risk of cervical cancer and the prevalence of VLP ELISA positivity for several populations.

We have analyzed the cellular effects of E2F-1, a transcription factor that is released from pRb when it binds to HPV16 E7, which is selectively retained and expressed in genital HPV associated cancers. An E2F-1 cDNA was toxic to keratinocytes and fibroblasts when expressed as high levels and this toxicity required the DNA binding but not trans-activating function of E2F-1. At lower levels, E2F-1 could functionally substitute for a pRB binding defective mutant of E7 but not for E7 entirely in the keratinocyte immortalization assay. Therefore, it appears that limited activation of E2F-1 is an important, but not the sole, function of E7.

Major Findings: ZO1 CB 09052-06 LCO

Background: Papillomaviruses (PVs) infect the epithelia of a wide variety of animals and man where they generally induce benign proliferation at the site of infection. In some cases, however, the 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 recent goals of the laboratory have included elucidating the mechanisms by which the viral oncoproteins induce cell proliferation and transformation, developing methods to facilitate the identification high risk HPV infection and thereby individuals at risk for developing cervical and other cancers, and generating immunogens that could ultimately be used in the development of a safe and effective vaccine to prevent genital HPV infection.

1. Identification of authentic wild type HPV16 L1 protein. We recently demonstrated in a recombinant baculovirus insect cell system that BPV L1 protein self-assembled into properly folded virus-like particles (VLP). However, when this approach was carried over to HPV16, which is the PV type most frequently found in cervical cancer, L1 from the prototype HPV16 strain that is used by virtually all investigators self-assembled three orders of magnitude less efficiently than that of BPV L1. This difference was shown to be inherent to the prototype L1; we found the L1 protein from HPV16 strains isolated from condyloma acuminata self-assembled as efficiently as BPV L1. Sequence analysis revealed that the prototype L1 contained a point mutation in a conserved amino acid, which accounted for its poor efficiency of self-assembly.

2. Seroreactivity of women in an HPV16 virus-like particle ELISA correlates with cervical cancer risk. There is currently no useful serologic assay for benign or pre-malignant HPV infection. Using the newly identified wild type HPV16 L1 protein described above, we have developed on ELISA based on self-assembled HPV16 VLPs, which, in a recently published study, detected IgG serum antibodies in the majority of women who had tested positive for HPV16 DNA. The antibodies are directed against conformational epitopes present in the wild type VLPs but not in the prototype HPV16. We have now used the ELISA to test groups of Spanish, Colombian, Danish, and Greenland women and have determined that there is a strong association between the prevalence of antivirion antibodies and cervical cancer risk in these populations. Women with HPV16 associated premalignant and malignant lesions in Colombian and Spain were similar in seroreactivity in the ELISA (70% for CIN III and 50% for cervical cancer). In contrast, 43% vs 3% of CIN III controls and 16% vs 1% of cancer controls were seropositive in Colombia and Spain, respectively. The results for the controls correlates with the eight fold higher incidence of cervical cancer in Colombia. Cervical cancer rates are also very high in Greenland, yet several surveys of HPV DNA have not found a high prevalence among Greenland women. In contrast we have found that 65% of a representative group of Greenland women were ELISA positive, suggesting that, for some populations, the ELISA may be a more accurate means of assessing risk of HPV associated cervical cancer. Preliminary analysis of sera from women enrolled in a prospective case-cohort study of HPV associated cervical disease suggests that the ELISA may detect more than 80% of women who are persistently infected with HPV16 but who do not yet have severe cervical dysplasia. 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 prove useful as an adjunct to Pap screening to identify women at risk for developing cervical cancer.

3. A virus-like particle vaccine protects rabbits from experiment CRPV infection. We have previously demonstrated that rabbit sera raised against BPV1 VLPs contained high titers of antibodies that were able to neutralize BPV infection of cultured cells. To

explore the potential utility of VLPs as a prophylactic vaccine, we have tested the ability of CRPV and BPV1 VLP preparations to prevent experimental infection by CRPV in rabbits. CRPV L1 or L1/L2 VLPs, in either Alum or Freund's adjuvent, induced full or partial protection against experimental challenge by high dose CRPV virus. In contrast, vaccination with BPV1 VLPs provided no protection. These results demonstrate the potential efficacy and type specificity of this type of vaccine.

4. Analysis of the interaction of papillomavirus with cell surfaces and relationship to antibody neutralization of viral infectivity. We have begun to explore the interaction between PV and cell surface receptors, which have not been identified for PVs. Radioiodinated BPV virions bound cell monolayers in a dose dependent manner. Both binding and BPV induced focal transformation were effectively blocked by BPV L1 or L1/L2 VLPs or the VLPs of HPV11 and HPV16, indicating that L1 is the major determinant of binding and that the three viruses most likely interact with the same cell surface receptor. BPV virions bound a diverse set of cultured cells, suggesting that this receptor has a wide tissue distribution and is conserved in evolution. Polyclonal sera raised to intact, but not denatured, BPV virions or L1 VLPs inhibited binding and infection of BPV. Sera to intact HPV16 VLPs were not inhibitory, demonstrating the type specificity of neutralization. Analysis of a set of neutralizing monoclonal antibodies that recognize conformational epitopes on BPV L1 indicated that neutralization can occur by two distinct mechanisms: blocking cell surface binding and blocking a later step. Radiolabeled HPV16 VLPs also bound to cell surfaces. Rabbit sera raised against wild type HPV16 VLPs blocked binding, while sera to the HPV16 prototype that self-assembles inefficiently, did not block binding. These results suggest that the wild type HPV16 L1, but not that of the prototype strain, may be effective in inducing neutralizing antibodies and therefore form the basis of a prophylactic vaccine against HPV16.

5. Analysis of E2F-1 activity in relationship to cell proliferation and HPV oncoproteins. Binding HPV16 E7 to the tumor suppressor protein pRB causes the release of the active form of the transcription factor E2F, which regulates the expression of genes involved in cellular proliferation. An E2F-1 cDNA was unable to fully substitute for E7 in a human keratinocyte immortalization assay, but it could, if expressed at low levels, complement the E7 mutant p24GLY, which does not bind pRB. However, when expressed at low levels E2F-1 or E7 can transform NIH 3T3 cells to anchorage independent growth. In contrast, high levels of E2F-1 inhibited the growth of keratinocytes and several established lines, as measured by the reduction of colonies obtained after co-selection with G418. This inhibition appears to be independent of p53, since cotransfection of human primary keratinocytes with the E2F-1 gene and HPV16 E6, which targets p53 for ubiquitin mediated degradation, or a dominant mutant p53 gene did not rescue the cells from growth inhibition. Analysis of a series of E2F-1 mutants showed that growth inhibition required the sequence specific DNA-binding activity but, surprisingly, not the transactivation or RB binding activities of the protein. Therefore, high levels of active E2F-1 are inhibitory to cell proliferation, while low levels play an important role in mediating HPV16 E7 induced growth stimulation.

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SUMMARY REPORT

LABORATORY OF GENETICS, DCBDC, NCI

October 1, 1993 through September 30, 1994

The mouse plasmacytoma (PCT) system is widely used by investigators in the Laboratory of Genetics to study pathophysiological mechanisms in tumor development. A major interest is in identifying genes that determine the unique susceptibility of BALB/c mice to this form of tumor induction. Two participating genes have been localized to separate regions on chromosome 4. We are still trying to place these genes in recombinational units of less than 5 centimorgans and are making progress in this effort. Crucial, though, is defining the action of these genes and the development of a quick assay. A potentially important clue provided by the PCR detection of myc-Sα illegitimate recombinations is that there are quantitative differences in the number of these recombinations between BALB/c and the partially resistant C.D2 MIA strain as shown by Jürgen R. Müller. This provides a basis for a 30 day test and suggests the chromosome 4 genes act much earlier than previously suspected.

The study of silicone gels as inducers of plasmacytoma formation has opened new questions and possibilities. First, silicone gels are as effective as pristane; the chemistry of silicone gels is more complex. We are attempting to identify the active components in the gels and determine if their primary effect is due to their immunological adjuvanticity or to direct genotoxicity. Because of the wide-spread use of these gels in implants, this work may be relevant to the study of the pathogenesis of multiple myeloma.

Dr. Beverly Mock is continuing to position chromosome 4 PCT susceptibility genes using the panel of typed backcross DNAs. She also has found genetic differences that influence PCT induction by the RIM (myc-Ha-ras) and ABL/MYC (myc-abl) retroviruses in pristane-conditioned mice.

Dr. Siegfried Janz's work seeks to elucidate underlying mechanisms in the formation of illegitimate recombinations. He and Jürgen R. Müller have found that these translocations are associated with deletions in c-myc and, further, that there are striking differences in length of these deletions in established PCTs and those found in c-myc-Sα recombinations at 30 days. In the established tumors the deletions are much more extensive. This suggests a cell selection process occurs or that the break sites occurring in a cell are unstable during tumor development. Dr. Janz has also found that c-myc rearranges with other genes.

Dr. Jürgen R. Müller has developed a sensitive nested PCT assay for the human c-myc-Sα illegitimate exchange and identified evidence for the translocations in HIV^+ men.

Dr. Francis Wiener has found that trisomy 11 is a consistent marker for ABL/myc plasmacytomagenesis. Through the ingenious use of critical chromosomal markers, he has located the genetic region which contributes to this form of plasmacytomagenesis on the telomeric end of chr 11.

Dr. Konrad Huppi is continuing to study the very complex and difficult problem of the Pvt-1 locus. Pvt-1 is located 3' of c-myc and is the target for chromosomal translocations in both humans and mice. These translocations do not disrupt c-myc but rather de-regulate its transcription through an as yet undefined process. The Pvt-1 translocation is a model for other translocations that affect distant oncogenic targets. Dr. Huppi has shown that Pvt-1-IgL translocations lead to the transcription of a chimeric RNA. The role of this RNA in deregulating c-myc transcription is being explored. The Pvt-1 locus is inefficiently repaired.

Dr. J. Frederic Mushinski's section is continuing to study the role of PKC isoforms in cell differentiation and proliferation. The transcription of cyclin genes B1, D1, D2 and D3 in B cell tumors is being evaluated. Dr. Mushinski is continuing to develop the ABL-MYC retroviral method for producing monoclonal antibodies. This system provides an alternative to the Kohler-Milstein hybridoma method.

Dr. Stuart Rudikoff's laboratory continues to study mechanisms in plasmacytomagenesis induced by the J3V1 (raf-myc) virus. They have shown that BALB/c nu/nu mice developed B cell lymphomas rather than PCTs. T cell reconstituted nu/nu mice developed PCTs, suggesting T cells play a role in driving the cells to the plasma cell stage during the transformation process. In histochemical studies of pristane plasmacytomagenesis, lymphoid aggregates in the oil granuloma are being analyzed to determine clonability of these populations and their relationship to subsequent plasmacytomas.

Dr. Linda Wolff continues to develop her intriguing model system of myeloid tumorigenesis and has been examining the genetic basis of susceptibility to a MuLV-induced myeloid leukemia (MML) in mice. She has identified strains that are susceptible or resistant to the induction of this tumor. The c-myb gene is the target of an initiating oncogenic mutation. Her recent evidence indicates that a non-MHC T cell immune response is responsible for eliminating pre-leukemic cells with these mutations in resistant strains. MML has also been induced in BALB/c nu/nu mice, but these myeloid leukemias do not have the typical effects seen in MMLs induced in intact mice involving the 5' end of c-myb. Dr. Wolff is attempting to determine how mutations of c-myb affect granulocyte proliferation and differentiation.

Dr. Wendy Davidson is making progress in unravelling the complex biology of lymphoproliferative diseases in mice that are associated with autoimmune phenomena. Inherited mutations in two lymphoproliferative gene loci in the mouse, lpr on chr 19 and gld on chr 1, result in a similar lymphoproliferation of massive proportions. The two genes code for a receptor and a ligand called FasR and FasL. The predominant cell type that accumulates in these lymph nodes is a $B220^{+}CD4^{-}CD8^{-}$ T Double Negative (DN) T cell and a minor population of $CD4^{+}B220^{+}$ T cells. Dr. Davidson has developed data that supports

the origin of DN cells from positively-selected $CD8^+$ T cells. The FasR and FasL genes regulate the numbers of peripheral T cells (predominantly $CD8^+$ T cells), and a defect in these genes leads to the accumulation of the DN cells.

Dr. Smith-Gill is continuing to work on the structural and physical-chemical basis of protein-antibody interactions to define principles that will be useful in designing antibody molecules and vaccines. Lysozyme-monoclonal antibody Fab complexes made with site-directed mutants of HEL or the mAb are crystallized and their structures determined. New work is directed toward exploring the mechanism of antigen presentation by B cells. Dr. Smith-Gill is beginning to develop a new area of research relating to mammary tumorigenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05553-25 LG

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	N. Swaminathan	Visiting Fellow	LG, NCI

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LAB/BRANCH

Laboratory of Genetics

SECTION

Molecular Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8	6	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. Susceptibility to plasmacytoma development in the BALB/c mouse has previously been shown to, in part, be determined by the genotype of the BALB/c B cell. However, the role of other cell types in this neoplastic process is largely unknown. To examine the potential contribution of T cells in tumor progression, we have employed the *raf-myc* containing J3V1 retrovirus to induce lymphoid tumors in both BALB/c and T cell deficient BALB/c nude mice. Tumors induced in BALB/c mice are almost exclusively plasmacytomas, whereas those arising in nude mice are predominantly B cell lymphomas. To define the role of T cells in this progression, nude mice were reconstituted with purified T cells and subjected to tumor induction. Tumors arising in T cell reconstituted nude mice were predominantly plasmacytomas with no significant differences seen between these animals and normal, immunocompetent BALB/c mice. These results indicate that the transformed cell in this progression is a B cell which is driven to terminal differentiation by peripheral T cells. This observation appears to correlate strongly with the clinical situation found in human myeloma where circulating, abnormal B cells have been observed which are clonally related to the malignant plasma cells found in the bone marrow. Our results suggest that these B cells may be driven to terminal differentiation by circulating T cells and that therapy regimens may need to more fully consider the circulating B cell as a target. **II.** The evolution of pre-neoplastic lesions in the BALB/c granuloma has been assessed by *in situ* studies of the cellular composition of pristane induced granulomatous tissue. Aggregates of B cells are routinely detected as early as 30 days post pristane injection in local lymphatics. These aggregates are routinely associated with developing plasma cells which are mono or pauci-clonal as determined by hybridization with multiple Vh probes. Such clones are continually arising in the granuloma and the cells therein rarely undergo apoptosis nor do they express plasmacytoma specific genes isolated from subtractive libraries suggesting that they are not transformed. These studies have defined a novel physiological situation in which cells that would normally undergo apoptosis and be eliminated are allowed to survive in the stimulatory environment of the granuloma. The failure of such cells to undergo normal elimination allows this population an extended window in which to accumulate the number of mutations necessary for neoplastic transformation and overt malignancy. It remains to be determined whether cells in these clones have already undergone the *myc* translocation characteristic of murine plasmacytomas.

Major Findings

I. The prototypic susceptible and resistant mouse strains for plasmacytoma induction are BALB/c and DBA/2, respectively. In previous studies, we addressed questions of the cellular basis of plasmacytoma development by cell transfer experiments into SCID mice. Results demonstrated that the susceptible phenotype for J3V1 (raf-myc) retroviral induced plasmacytomagenesis was directly associated with the BALB/c B cell genotype. Recently, we have turned to the question of the role of other cell types in the initiation/progression of this disease. We have initially focussed on the role of T cells by performing induction experiments in BALB/c nude mice lacking functional T cells and nude mice reconstituted with purified T cells of BALB/c origin. Tumors obtained from these animals have been compared with those from normal, immunocompetent animals where the J3V1 virus induces mature plasma cell tumors that secrete Ig and express the cell surface antigen ThB. Approximately 20% of these co-express the B cell form of the CD45 antigen (B220). In contrast, B lineage tumors arising in T cell deficient nude mice rarely express the mature plasma cell phenotype (17%). Most tumors express high levels of B220, do not secrete Ig and are characterized as B cell lymphomas.

To assess the possible role of T cells in tumorigenesis, nude mice were reconstituted with purified BALB/c T cells and subjected to tumor induction. Analysis of 24 B lineage tumors arising in T cell reconstituted nude mice reveals that 67% (16/24) express a mature plasmacytoma phenotype while the remaining 33% (8/24) are typical B cell lymphomas. The frequency of mature plasmacytomas in reconstituted mice is significantly different ($p = 0.0002$) from that observed in untreated nude mice, but not significantly different ($p = 0.60$) from that observed among immunocompetent animals. Thus, our data clearly suggest that T cells, while not necessary for J3V1-induced transformation, provide signals that are required for the differentiation of transformed B cells into mature plasma cells. We believe this finding is particularly relevant to human myeloma where circulating B cells which have an abnormal phenotype have been shown to be clonally related to malignant plasma cells found in the bone marrow. Our data in the murine system thus suggest that the transformed cell is likely to be a B cell which is driven to terminal differentiation by signals received from peripheral T cells. This observation is pertinent to the design of appropriate treatment regimens for multiple myeloma as targeting the plasma cell population without concomitant elimination of any transformed B cells may clearly lead to subsequent relapse.

II. Plasma cell tumors are the end product of a neoplastic process about which surprisingly little is known in terms of early cellular events. To examine early cellular aspects of the disease process, we have developed *in situ* procedures to characterize cellular dynamics in the pristane induced oil granuloma. These studies have revealed a novel lymphoid compartment which may be a source of B cells eventually entering the peritoneum. Using an antibody to the B cell marker CD45R (B220), a population of positively staining cells has been identified in the lamina propria lacteals of pristane treated mice which is absent in untreated animals. These cells are not undergoing apoptosis and may play a role in antigen recognition and the gut immune response

as well as possibly being a source of B cells which are targets for transformation in the oil granuloma.

In situ analysis of lymphoid populations in the pristane induced oil granuloma has elucidated the presence of B cell aggregations in local lymphatics. These cells are not undergoing apoptosis and appear to give rise to associated plasma cells after leaving the lymphatics. One to several clones of plasma cells are frequently observed in association with a single lymphoid accumulation. These structures of B cells and associated plasma cells can be found from 30 days post pristane until the observation of a frankly malignant tumor. The clonality of lymphoid associated plasma cells has been assessed by *in situ* hybridization with multiple Vh probes. The developing plasma cell clones are generally monoclonal with a number of unrelated clones developing simultaneously. This suggests that plasma cell clones are continually arising in the oil granuloma and persist for significant lengths of time thus presenting a novel population of cells which are likely the target for mutational events. These clones do not express the plasmacytoma - specific gene pc326 (identified by Dr. M. Keuhl) which we have found to be expressed in primary plasmacytomas and appear not to be fully transformed. However, the failure of such clones to undergo apoptosis increases the probability that they will accumulate the number of genetic changes necessary for neoplastic transformation and suggests they are precursors of the eventual neoplastic cell.

III. In the previous annual report, we described the dependence of *in vitro* growth of primary murine plasmacytomas on the presence of stromal cells from the site of the developing plasmacytoma. A series of rat hybridomas to these stromal cells has been generated and is currently under investigation. To date, twelve of these which react strongly with granuloma tissue have been characterized as to tissue staining patterns on frozen and paraffin sections. All appear to be directed toward endothelial elements indicating this is the major source of stroma in the granuloma. Several patterns of reactivity have been identified on normal tissues. The most interesting of these are antibodies which stain the glomerulii in the kidney and capillaries in other organs but do not react with larger blood vessels. A second set does not stain the glomerulii, but reacts with blood vessels at other sites. Studies are currently underway to identify and characterize the molecules recognized by these antibodies.

IV. As part of our effort to establish a mouse model for gene therapy of myeloma, we have initiated experiments to evaluate the metastatic potential of murine plasmacytoma. One of the fundamental differences between the murine and human forms of this disease is the localization of murine plasmacytoma to the peritoneum as opposed to the bone marrow as seen in human disease. Initially we have evaluated the ability of established murine plasmacytoma lines to leave the peritoneum, enter the circulation, and become established at distant sites. Two such lines were both found to locate in distant organs indicating an ability to both enter the blood stream and subsequently extravasate into tissues. When injected intravenously, one of these lines displays a metastatic pattern similar to that seen in late stage human myeloma. Metastases are found in the bone marrow, spleen, lungs, liver and meninges of the brain. We are currently addressing the question of whether earlier granuloma or IL-6 dependent tumors will exhibit similar properties.

Based on the above results, we are developing strategies for a gene therapy model. It has previously been demonstrated that introduction of the IL-2 or IL-4 gene into myeloma cells and their subsequent inoculation into either normal mice or animals with unmodified tumor cells, leads to protection. In these instances, the tumors were evaluated only at subcutaneous sites. To extend these studies, we have generated an IL-2 containing retrovirus for infection of the lines with metastatic potential. Using the above system, we will be able to evaluate such an approach on localized as well as disseminated disease.

V. Analysis of B cell lymphomas induced by J3V1 virus in nude mice has yielded several tumors of particular interest in that cell surface molecules can be regulated by specific cytokines. Cell lines have been established from two of these in which CD45R (B220) expression can be upregulated by either IL-6 or IL-4 and the regulation is reversible upon removal of the cytokine. The mechanism of CD45 regulation has been investigated by the use of monoclonal antibodies specific for particular exons as well as PCR technology. Using these two approaches, it has been determined that CD45R (B220) up-regulation in response to either IL-4 or IL-6 is due to a change in the splicing patterns of the native CD45 transcript rather than changes in the overall expression of the gene. In cells grown in the absence of IL-6 the variable exons 4, 5, and 6, are spliced out, while those cells grown in the presence of cytokine fail to remove the variable exons. Analysis of CD45 intron and exon sequences suggests that the alternatively spliced exons are flanked by cryptic splice recognition sequences that are recognized less efficiently than those flanking the constitutively expressed exons. We are currently designing episomal DNA vectors to monitor splicing and to aid in the identification of sequences controlling the choice of alternative splicing patterns in the CD45 gene. Since the observed splicing is essentially quantitative, this system provides one of the best examples of mammalian alternative splicing yet identified for exploration of this process.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05596-25 LG

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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SECTION

INSTITUTE AND LOCATION

NCI, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
14	5	9

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The last year has seen substantial progress in the mouse plasmacytoma model system. First, silicone gels have proven to be highly effective inducers of plasmacytomas, and this chemically defined but complex material opens new approaches to understanding how agents that induce chronic granulomatous responses are related to the process of plasmacytomagenesis. In contrast to pristane, the silicone gels are well tolerated and produce few side effects, permitting more accurate determination of plasmacytoma incidence with fewer mice. Second, many of the technical problems in carrying out reliable nested PCR amplifications of the c-myc-Sa illegitimate recombinations have been worked out, and this can now be used as an assay for chromosomal translocation and assessing the role of genes in determining this oncogenic mutational process. Recombinant BALB/c.DBA/2 chromosome-4 congenic strains with even smaller regions of DBA/2 chromatin are now available and under analysis for susceptibility and resistance to plasmacytoma induction. Using reliable cytofuge preparations has made it possible to determine the susceptibility/resistance status of many of our congenic lines. An investigation of genes on chromosome 11 continues. BALB/c congenic mice carrying the xid mutation are clearly resistant. With Juergen Mueller and Charles Rabkin, a PCR methodology for assaying the human t(8;14) translocations involving c-myc-Su recombinations has been established, and the peripheral blood lymphocytes from 127 HIV+ men are being analyzed; translocations have thus far been detected in 5 of these. The work indicates that the c-myc-Ig switch region illegitimate recombination is an early oncogenic step that is required but not sufficient to transform a B lymphocyte to full malignant potential.

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

Genetic factors in the BALB/cAnPt colony

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 60-30% 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 certain C.D2 plasmacytoma susceptible congenics, notably C.D2-Idh1-Pep3 (chr 1), C.D2-Es3-Hba (chr 11), C.D2-Qa2 (chr 17), and C.D2-Rmcfr/r (chr 5), which have been used in coincidental studies, have given the expected high incidence of plasma cell tumors. We are now using the C.D2-Idh1-Pep3 strain as the test strain of choice for PCT induction studies.

An analysis of 4 sublines of BALB/cAnPt (A, B, C and D) continues. Further, we continue to get erratic yields of PCTs using the standard "A" subline ranging from 30-50%. In contrast, C.D2-Idh1-Pep3 gives high yields approaching 80%. This problem has held up progress for several years, and it is hoped that progress towards its resolution will be forthcoming with one of the new sublines currently under test.

Environmental factors

Previous work has shown the requirement for a conventional environment for plasmacytoma induction by pristane and that only an extremely low incidence of these tumors can be induced in Specific Pathogen Free BALB/cAnPt mice. We have not identified the specific factors in the conventional environment. Our conventional colony harbors both Sendai and Mouse Hepatitis Viruses. Early infections with these agents may be essential in 'tuning' the immune system. We have noted in the past some anecdotal associations of plasmacytoma development and outbreaks of mild Sendai and MHV disease. Both diseases occur mildly and sporadically. An experiment is underway to explore the role of litter seriation on subsequent susceptibility to plasmacytoma induction. It is proposed that the first litters will be more prone to developing infections with Sendai Virus and, hence, have immune systems that are more active than mice obtained from later litters where the mothers are now solidly immune to both Sendai and MHV.

Plasmacytoma inducing agents: plastic discs

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. Fortunately, a large part of our experiment was set up in BALB/c.DBA/2-Idh1-Pep3 mice, and tissues from 44 mice in various stages of solid plastic- induced PCTs have been collected. These tumors appeared to develop in the connective tissues of the omentum. Milky spots (lymphoid aggregates) in the omentum are

in some histological sections associated with early plasmacytoma formation. The paucity of fibrocollagenous reactive tissue that forms on other peritoneal surfaces strikingly contrasts to the oil granuloma. Chronic inflammation does occur in these mice and appears to be caused by the omentum wrapping around and attaching to the disc. However, the adhesions are weak and probably through motion and peristalsis these junctions break, leaving tissue on the disc which becomes necrotic. This may provide a sufficient inflammatory process to permit PCT development. Evidence for this mechanism can be achieved by comparing the effects of polished versus rough-edged discs, as the omentum may not be able to adhere to the polished discs.

Plasmacytoma inducing agents: silicone gels

We have identified a new plasmacytomagenic agent, the silcone gel used in breast implants. The intraperitoneal injection of 0.4 ml or 0.1 ml of this very sticky material given three times at 2 month intervals 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 liquid material from the silicone gel has seeped out, become surrounded with inflamatory cells and conglomerates seeded onto mesenteric surfaces. This excites an angiogenic response from the mesenteric vessels beneath, which now leads to the formation of a chronic granulomatous tissue. The various focal points where the silicone granuloma (SilG) develops on the intestinal mesenteries are sites where the mesothelium balloons out to form an angiogenic mesothelial polyp.

The basic motif in the SilG is the highly refractile silicone vacuole. The SilG goes through 3 phases of development: 1) inflammatory, which lasts for an extensive period of time and is associated with a substantial number of chronic inflammatory cells between the silicone vacuoles; 2) excessive collagen formation, and 3) burned out phase. The BALB/cAnPT"A" mice have, however, developed plasmacytomas which are associated with this granuloma tissue which compares well with the yields we obtain with pristane. The C.D2-Idh1-Pep3 mice develop high incidences of PCTs (near 80% in some experiments) at 300 days. These mice are now being used as the test strain to evaluate various components of the silicone gels.

We have begun studying the components of the silicone gels. Silicone gels are made by crosslinking co-polymers of methylhydrogenpolysiloxane with vinylmethylpolysiloxane by using a powerful reducing agent, platinic chloride. The crosslinking process is incomplete, which is essential for preserving the appropriate consistency of the gel, and this leaves unreacted vinyl groups in the gel. Some of these can be on crosslinked chains; others are on liquid linear and cyclic compounds that are trapped in the gel matrix. We have tested the liquid forms of dimethypolysiloxane of 1,000 and 12,500 cst viscosity, and these have not yet produced PCTs. Currently under test in C.D2-Idh1-Pep3 are: 1000cS vinylmethyl-polysiloxane with and without added platinic chloride, gels from 2 other implants and gels made in the laboratory from the Huls America kit. In addition, it has been possible to make

liquid emulsions of 1000 cS DMPS with various gel preparations. These have been shown by others to be excellent immunological adjuvants, and hopefully a standard, easily injectable preparation can be found that will replace the silicone gel and pristane. Thus far, the important characterisitic of the silicone gels is that they appear to slowly release liquid materials. The continuous availability of granuloma formation may be an important factor in recruiting available B cells with c-myc-Sα recombinations. The possible direct role, however, of specific silicone gel derived chemicals in this process is being explored. A document released by Dow-Corning on the biological effects of siloxanes indicated that the 4 silicon unit, the D4 compound octamethyltetrasiloxane, was biologically active and activated macrophages to release gamma interferon. We are testing this compoound, as well as the companion, vinylmethytetrasiloxane, to determine if these compounds can be degraded to yield low molecular weight vinyl compounds that can be further metabolized to yield electrophilic agents that can attack DNA.

The silicone gels are possibly relevant to human plasma cell tumor development, as these materials are found in mammary implants which have between implanted in 1 to 2 million women in the US. It has been estimated in the JAMA article that around 4-5% of these implants leak or that low molecular weight materials seep through the capsules. In humans silicone granulomas form around the capsules. While local plasmacytomas have not been reported, the more important question is whether the inflammatory tissue site could be a participant in PCT development. Possibly, local granulomas forming around leaky implants may not have the appropriate vasculature for PCT development, but these sites may attract other B lymphocytes and interact with them. Cells migrating through these chronic inflammatory sites might subsequently home to the bone marrow, which is regarded as the tissue site of origin of MGUS and Multiple Myeloma, the most common forms of plasma cell tumor in humans. It also remains to be determined if liquid silicones derived from leaky implants themselves could migrate to the bone marrow where MGUS and Muliple Myeloma develop in humans. Very little is known about granuloma formation in human bone marrow and its potential role in tumor development. Second, the silicone gels may act very non-specifically as chronic immunological adjuvants. This may stimulate B lymphocytes to proliferate and increase the chances for the development of critical oncogenic mutations. An active epidemiological study of women with breast implants should be undertaken to determine if they have increased numbers of expanded B cell clones (MGUS) or myeloma proteins. Thus far, several anecdotal reports mentioned at scientific meetings have noted the occurrence of myeloma in women with implants. These cases must be carefully collected and documented before any interpretation can be made.

Genetic studies

The major effort is to identify the genes on chromosome 4 that are play a role in PCT susceptibility. Two genes, the centromeric PctR1 which is located near the Ifa locus and the PctR2 gene located near Fv-1, have been identified only through linkage relationships. The presence of only one of these PCT-R genes can produce a significant resitance to plasmacytoma induction. This suggests that susceptibility requires the cooperation of at least

2 PCT-Susceptibility genes. The congenic strain C.D-2-TF3 carries the PctR1, while the C.D2-Fv-1 carries the PctR2 gene. The C.D2-MIA congenic has both genes. These mice are very useful in looking for relevant biological phenotypes that have the DBA/2 type response. All three of the above mentioned strains develop very reduced, but not negligible, incidences of plasmacytomas following standard induction protocols. Starting with these 3 congenics, further backcrossing has been carried out to eliminate more DBA/2 marker segments that are not involved in plasmacytomagenesis. We are currently testing many of these second generation recombinant congenics for plasmacytoma incidence.

The particular value of the C.D2 congenics is for identification of a relevant phenotype. One hypothesis that has been pursued for some time in the laboratory has been that BALB/c mice are genetically prone to generating illegitimate recombinations [i.e., the t(12;15) and t(6;15) chromosomal translocations] that activate c-myc.

In collaboration with Siegfried Janz, Jürgen R. Müller and Gary Jones, the oil granuloma tissues at day 30 post pristane are being assayed using nested PCR for the occurrence of c-myc-Sα illegitimate recombinations. By day 30 junction fragments can be found in 50-70% of C.D2-Idh1-Pep3 and BALB/c mice. A striking finding made now in 2 separate experiments has been the finding that less than 10% of C.D2-MIA mice carry these illegitimate recombinations. This evidence suggests that the chromosome 4 DBA/2 genes are in some way inhibiting the development of these recombinations.

Search for additional resistance genes

We are continuing to develop and characterize the many other C.D2 congenic mice to search for additional genes that determine resistance to plasmacytomagenesis. Most emphasis has been placed on chromosome 4 genes. Several years ago Beverly Mock tested the C.D2-Es3-Hba congenic and found it to be resistant. Subsequent tests have shown it to be highly susceptible. The Es3 and Hba are distantly located on chromosome 11, and both markers were used in the development of the congenic strain in the hope that the entire DBA/2 chromosome would be introduced. Apparently, at the 10th backcross generation when the mice were being made homozygous for Es3 and Hba, the early homozygotes contained two forms of chr 11; one had undergone a double crossover to eliminate the central segment, while the other was intact. The presence of a resistance gene in this central segment has been postulated. Unfortunately, the current C.D2-Es-Hba lacks the central DBA/2 derived segment and is highly susceptible. Accordingly, new chromosome congenics carrying this region are being constructed. The emphasis on chr 11 is due to the presence of many potentially interesting genes which have been implicated in other disease processes. Cory Teuscher at the University of Utah is helping with the characterization and development of these developing congenics.

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. The final induction study with these mice has been a comparison of the males and females derived from the cross C.D2-Idh-Pep3(+/+) X C.xid/xid. The male progeny are phenotypically immunodeficient, while the female heterozygotes are normal. Thus far, we have obtained PCTs in the females and not in the males.

We are pursuing this further in collaborative experiments with Francis Wiener in which BALB/cAn.CBA-xid/xid mice will be given cytogenetically normal BALB/c cells to determine if both the peritoneal B population and plasmacytoma susceptibility can be restored.

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.

Detection of t(8;14) translocations by PCR in pre-neoplastic states

In collaboration with Jürgen R. Müller, Charles Rabkin and Siegfried Janz, a series of PCR primers has been developed to detect illegitimate recombination fragments from c-myc to switch mu ($S\mu$). This has proven to be highly successful in generating fragments from 14 of 15 known sporadic Burkitt's lymphomas. The recombination structures from both of the reciprocal chromosomes were recovered and sequenced. The analysis of these recombinations showed that the joining process was nearly reciprocal; however, deletions of 4 to 69 base pairs was found in c-myc. This contrasts sharply from the results with developed mouse PCTs where deletions in c-myc are frequently as long as several hundred base pairs. It has been hypothesized that switching enzymes may play an important role in this process as pentamers resembling the repeat units in $S\mu$ are found in c-myc.

Nested PCT amplification has been used to search for these t(8;14) recombinations in HIV^+ men. DNA was extracted from PBL lymphocyte samples. Although the study is still in progress, 3 recombinations have been found in 43 subjects who do not have evidence of

lymphoma. One of these had recombinations detectable over a 7 year period; there were 2 different recombinations in this individual. Eleven of the subjects have developed lymphoma during the course of the study. Two of these had c-myc-Sμ recombinations a year before the tumor. Analyses of the lymphomas has not yet been carried out.

II. ABL-MYC Plasmacytomagenesis - Dr. Francis Wiener

Plasmacytomagenesis in SCID mice

The inability of the SCID mouse to develop functional B cells that can undergo switching is a dominant feature of the SCID phenotype. Consequently, plasmacytomagenesis is virtually abrogated in this strain in spite of its susceptible BALB/c background. We asked the question whether plasmacytomas (PCTs) could develop from the "leaky" B cell population since these cells are functionally similar to that of BALB/c-derived B cells. Repeated attempts to generate PCTs by conventional induction methods failed, suggesting the "leaky" B cells may lack the mechanism for MYC activation by chromosomal translocation.

To test this hypothesis we induced PCTs in young (2 months of age) and old (5 months of age) groups of SCID mice by ABL-MYC retrovirus that obviates the requirement of chromosomal translocation. IgA, IgG and IgM producer PCTs developed in both groups with an incidence of 10% and 25%, respectively. The results suggest that the "leaky B" cells are able to develop into PCTs but probably are incompetent to deregulate MYC expression by chromosomal translocation. Alternatively, the simultaneous occurrence of two events, as the emergence of a "leaky B" cell clone which have undergone MYC translocation, appears unlikely.

The role of trisomy 11 (Ts11) in murine plasmacytomagenesis

Previously we have shown that Ts11 is the second most frequent non-random chromosomal change in murine plasmacytomas. The frequency of Ts11 is significantly higher in PCTs induced in pristane-conditioned mice infected by Abelson-MuLV compared to those induced by pristane alone. The regular presence of Ts11 in pristane + A-MuLV induced PCTs suggest that the overdosage of a gene or genes located on chromosome 11 may specifically act on the development of PCTs in which both oncogenes, MYC and v-ABL, are abundantly expresssed.

To test this assumption we induced PCTs by three highly effective plasmacytomagenic retroviruses: ABL-MYC, J3V1 and RIM. More than 80% of PCTs that arose in BALB/c, (BALB/c x DBA/2)F1, and 5 month old SCID mice infected with ABL-MYC virus were trisomic for chromosome 11. In contrast, 90% of PCTs induced by J3V1 or RIM retroviruses expressing v-MYC and raf-gag fusion protein or c-MYC and v-Ha-Ras oncogenes were Ts11 negative.

We have also investigated whether the whole or any particular region of chromosome 11 needs to be duplicated in the process of ABL-MYC induced plasmacytomagenesis. By inducing PCTs in F1 heterozygous mice which carry a Robertsonian or reciprocal translocation involving chromosome 11, we defined that the duplicated chromosomal segment is located distal to T53Dn breakpoint (11E1 sub-band) on the telomeric end of chromosome 11. We surmise that the regular duplication of this chromosomal segment indicates the presence of a gene with a critical role either in transformation of PCT precursor cells and/or in clonal development of murine plasmacytomas.

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

The inheritance of susceptibility to pristane-induced plasmacytomagenesis has been examined in backcross progeny generated from crosses of BALB/cAnPt (Pct-susceptible) and F1 hybrids (Pct-resistant) between BALB/c and DBA/2 strains of mice. RFLP analyses of the susceptible backcross progeny for a series of 3-4 markers per chromosome has indicated linkage of the susceptibility phenotype to mouse Chromosome 4. Restriction fragment and simple sequence length polymorphism analyses of a series of 30 markers distributed across the length of Chromosome 4 were completed on a series of 77 susceptible and 68 resistant backcross progeny. These analyses positions the susceptibility locus/region in the distal part of mouse Chr 4 with peak LOD (logarithm of the odds) scores near the markers *Scl (Tal2)*, *Gt-10* and *Tnfr2*. We are continuing to examine new markers from Chr 4 in an effort to saturate the regions linked to susceptibility loci. RFLP/SSLP analyses of resistant and susceptible congenic strains which are carrying recombinant genotypes across the distal portion of Chr 4 from *Ifa* to *D4Smh6b*, have confirmed that at least 2 genes (*Pctr1* and *Pctr2*) are linked to mouse Chr 4. These regions of mouse Chr 4 are 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. Efforts are concentrated on developing single stranded conformational polymorphisms and Northern analyses of candidate genes within the intervals surrounding the two resistance loci. 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). We have also positioned a series of 20 markers on Chr 1 near this susceptibility/resistance gene. Further analyses of the backcross progeny are being continued to provide complete coverage of the genome for markers linked to the susceptibility/resistance phenotypes.

Restriction fragment and simple sequence length polymorphism analyses of a series of congenic strains which carry DBA/2 alleles of genes across the distal half of the chromosome have narrowed the intervals surrounding *Pctr1* and *Pctr2* to approximately 5-7 cM each. We are utilizing congenic strains of mice which carry the resistance alleles of these two genes in the PCR-based subtractive DNA hybridization procedure referred to as representational difference analysis (RDA) to isolate new markers closely linked to the resistance genes. In addition, we are creating congenic strains which carry successively smaller regions of DBA/2 chromatin in the interval surrounding the resistance genes. Efforts are focused on producing

a fine structure genetic map of mouse Chr 4 for the careful delineation of the intervals surrounding the R/S genes.

We have also explored alternate methods of plasmacytoma induction which involve 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 J3VI 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.

In order to determine the chromosomal location of the susceptibility/resistance genes in the RIM and AM 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 Chrs 3, 4, or 11 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. In contrast, the C.D2-dC mouse which carries the DBA/2 allele of genes on Chr 9 is partially resistant to developing AM-induced plasmacytomas. In addition, 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. We are in the process of evaluating backcross progeny for associations of resistance/susceptibility phenotypes following RIM or AM induction of plasmacytomas with DBA/2 and BALB/c genotypes for markers on these chromosomes.

We have also completed the development of a series of bilineal C.D2-Chr 1 congenics which carry recombinations across the proximal and distal portions of the chromosome. These congenic strains were constructed for purposes of 1) evaluating the role of Chr 1 genes in plasmacytomagenesis, 2) fine-mapping the Rep1 DNA repair phenotype which we mapped to Chr 1 a few years ago, and 3) to produce the rough equivalent of a transgenic mouse for the *Lsh-Ity-Bcg* macrophage resistance gene, for which, the candidate gene, *Nramp*, was recently cloned. We have created a strain which carries less than 1 cM of DBA/2 (resistant) chromatin surrounding the *Nramp/Il8r/Vil* region of the chromosome on a BALB/cAnPt (susceptible) background. These strains are being utilized by a variety of investigators outside of NIH in their efforts to examine DNA repair phenotypes, cytokine profiles in response to intracellular microorganisms, properties of phagolysosomes of macrophages, and the growth and transformation characteristics of pre/pro-B cell lines.

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

We continued our studies on genetic rearrangements that activate the proto-oncogene *c-myc* in plasma cell tumor development in BALB/cAnPt mice. These rearrangements predominantly take the form of reciprocal chromosomal translocations t(12;15) that juxtopose *c-myc* to an immunoglobulin heavy-chain switch α (Sα) region. We employed PCR methods to analyze recombinations between Sα and *c-myc* as molecular indicators of t(12;15) and solidified our earlier observation that recombinations between Sα and *c-myc* occur with surprisingly high frequency, i.e., in 30-60% of the animals depending on the sensitivity of the PCR method being used, and very early in the preneoplastic stage of plasma-cytomagenesis, i.e., 7, 14, and 30 days post-pristane. Furthermore, the comparison of recombinations between Sα and *c-myc* in preneoplastic B cell clones with those seen in plasmacytomas revealed significant differences, suggesting that initial recombinations in preneoplastic B cells are distinct and either subject to remodeling or selection in the course of malignant transformation. These studies were mainly performed by Gary M. Jones and Jürgen Müller, who recently started to apply the experimental protocol developed in the mouse plasmacytoma system to the detection of the t(8;14) in Burkitt's and AIDS lymphomas.

The description of atypical *c-myc* activating rearrangements in some plasmacytomas by others encouraged us to test whether the diversity of *c-myc* activating recombinations could point to a hypothetical regional genomic instability of the *c-myc* locus in BALB/c mice. We applied PCR methods to look for non-conventional recombinations of *c-myc* in the preneoplastic stage of plasma cell tumor development and found recombinational fragments between *c-myc* and dispersed repetitive elements as well as *c-myc* and sequences 5' of Sα. Since we ultimately wish to determine the 5' limit of recombinations in the IgHα locus, we sequenced the region between Iα and Cϵ, thereby completing the sequence analysis of the entire IgHα locus. This information will be useful to test the hypothesis that the genetic basis for the prevalence of recombinations of *c-myc* with Sα rather than other switch regions may be related to a particular genomic instability of the IgHα region. Preliminary data show that intralocus IgHα recombinations are characterized by deletions and inversions and are more frequent in tumor susceptible BALB/cAnPt mice than in tumor resistant strains of mice. Klaus Felix has initiated experiments to test the relationship between genomic instability of B cells, as assessed by the frequency of intralocus IgHα recombinations, and the oxidative balance of B cells, which will be modified by depletion of glutathione.

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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, 1993 to September 30, 1994

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

Structure and function of oncogenes and anti-oncogenes

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

P.I.:	J.F. Mushinski	Section Chief	LG, NCI
	K. Huppi	Research Expert	LG, NCI
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COOPERATING UNITS *(if any)*

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Molecular Genetics

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8	3.5	4.5

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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.)*

Our research goal is to understand the molecular structure and function of the genes that play critical roles in malignancy, cell growth and normal differentiation. We are concentrating on a group of known oncogenes and anti-oncogenes: c-myc, abl, bcl-2, ras and p53, p21, p107, as well as potential oncogenes: Pvt-1, protein kinase C (PKC) and cyclins, in mouse and human tissues and tumors. Deregulated expression of c-myc secondary to chromosomal translocations in the c-myc region is known to be an essential element in the series of genetic alterations that are involved in plasmacytomagenesis in BALB/c mice and in Burkitt and AIDS-associated lymphomas in man. A "variant" subgroup of these myc-activating translocations occurs ~260kb 3' of c-myc in a region designated Pvt-1. We have shown that most of these variant translocations lead to the synthesis of a chimeric mRNA that includes a portion of Pvt-1 and the constant region of the kappa Ig light chain. This chimeric product may play a role in plasmacytoma development or in increasing c-myc expression. We have shown that DNA damage in the Pvt-1, c-myc, switch IgA and Ig kappa constant region genes is poorly repaired in strains of mice that are susceptible to plasmacytoma development, and these are the sites of recurrent chromosomal translocation in mouse and human B-lymphocytic neoplasms. There is a difference, however, between human and mouse tumors of this type, namely, human tumors have a high incidence of structural mutations in key onco-or anti-oncogenes, including p53, the ras family and c-myc, while these are rare in analogous mouse tumors.

A recombinant retrovirus that expresses v-abl and c-myc (ABL-MYC) rapidly induces plasmacytomas in 100% of BALB/c mice and transforms immature or mature B lymphocytes into malignant plasma cells. If the mice are immunized before infection with ABL-MYC, ~50% of the plasmacytomas produce antibodies that are directed toward the immunogen. This technology has been used to produce monoclonal antibodies to particulate, protein and peptide antigens, and it offers an alternative to hybridoma technology.

We have cloned eight isozymes of the PKC family into expression vectors and produced cell lines that overexpress each of these isoforms in NIH3T3 fibroblasts and in 32D promyelocytes. These overexpressing cell lines have proved useful for demonstrating isoform-specific characteristics: PKC-ϵ and -δ uniquely mediate 32D cells' ability to differentiate into macrophages when exposed to phorbol esters. Immunohistochemistry has shown that each isoform targets unique intracellular organelles when activated, suggesting that the substrate for each isoform is restricted to these sites.

Cyclins B1, D1, D2 and D3 have been cloned and used as probes to show that expression of these cyclins in hemopoietic neoplasms is cell-type specific. Regulation of cyclin D2 expression is being studied by a careful analysis of its promoter and upstream regulatory sites.

Other Cooperating Units within NIH.

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I. Magrath, K. Bhatia	PB	NCI
J.H. Pierce	LCMB	NCI
P.M.Blumberg, M. Kazanietz	LCCTP	NCI
A. Lewis, Y. Eyler	LIP	NIAID

Major Findings:

I. Overexpressed c-*myc* and v-*abl* from Infection with the ABL-MYC Retrovirus Rapidly and Specifically Induces Plasmacytomas. (Mushinski, Weissinger, Mischak)

Infection with ABL-MYC (a recombinant retrovirus that expresses v-*abl*)and c-*myc* provides the most rapid and specific way to induce plasmacytomas in 100% of BALB/c mice, even in the absence of helper virus or pristane. It also is effective in transforming B lymphocytes into plasmacytomas *in vitro*. If the mice are immunized before ABL-MYC infection, approximately half of the resulting plasmacytomas secrete antibodies to the immunogen. These immunogens may be red blood cells, carbohydrates, proteins or peptides. Many of the resultant plasmacytomas are monoclonal, thus this approach may offer a useful alternative to the conventional method of producing monoclonal antibodies.

These findings suggest that mature, committed B lymphocytes are the usual targets of ABL-MYC infection. Yet plasmacytomas resulted when bone marrow cells were infected with this virus, suggesting that earlier B cells might also be targets for ABL-MYC. This notion was confirmed by ABL-MYC infection of purified suspensions of pro-B cells (Whitlock-Witte cultures of bone marrow cells or day 12 fetal liver cells). Abelson murine leukemia virus induced only pre-B lymphomas in parallel cell preparations. These data indicate that constitutive expression of v-*abl* alone, via Abelson virus, transforms B cell precursors, but further differentiation of these cells is blocked. Addition of constitutively expressed c-*myc*, via ABL-MYC, abrogates this block and may even drive B cells to the plasma cell stage.

II. Constitutive Expression of c-*myc* in Atypical Plasmacytomas. (Mushinski, Shaughnessy, Wiener, Potter)

Plasmacytomas characteristically exhibit constitutive expression of c-*myc* that is secondary either to T(12;15) chromosomal translocations in the c-*myc* gene or, less often, T(6;15) or T(15;16) translocations in the Pvt-1 region (see section IV, below). There are, however, a few plasmacytomas that are exceptions to these rules, and the mechanism of c-*myc* activation was studied. ABPC 60 contains a T(12;15), but the c-*myc* locus is not involved. Cloning of the breakpoint in this tumor showed that it was located in the Pvt-1 region, 240-kb 3' of c-*myc*. This unique example of a T(12;15) breakpoint in Pvt-1, not c-*myc*, suggests that T(12;15)s and T(6;15)s may be fundamentally and mechanistically similar. That is, both may occur at the same stage of B-cell differentiation, and both may utilize the same recombination mechanism, probably that of the heavy-chain isozyme switch. ABPC 22 and RFPC 2782 are atypical in that they have no karyotypically discernible chromosomal abnormalities. Cloning of the region upstream from c-*myc* in these tumors showed that each had a retroviral provirus integrated in this region, the first reports of mouse B-cell neoplasms with c-*myc* activated by proviruses. In both cases the virus was inserted in a transcription orientation opposite to that of c-*myc*, indicating that the augmented c-*myc* expression came from the nearby viral LTR enhancer rather that its promoter. The ABPC 22 proviral enhancer was missing one of the two 72-bp repeats

RFPC 2782. This level of c-*myc* mRNA is uniquely low among all known plasmacytomas, but this level, expressed constitutively, appears to be sufficient to maintain the transformed state of this plasmacytoma.

III. Protein Kinase C: (Mushinski, Goodnight, Henderson, Mischak)

1. Increased expression of certain isozymes in lymphocytic tumors. We have produced full-length cDNAs for mouse PKC-θ and -ζ and probed blots of RNAs isolated from hemopoietic tumors. Isozyme-specific antibodies used on western blots confirmed the mRNA findings. PKC-θ is abundantly expressed in normal and transformed T, but not B, lymphocytes. On the other hand, PKC-ζ is found in abundance in neoplastic T and B lymphocytes but not in normal lymphocytes or in dividing or antibody-secreting splenocytes. Plasmacytomas had an unusually high amount of PKC-ζ mRNA, suggesting that this isoform may play a role in plasmacytomagenesis. We have constructed a PKC-ζ-expressing retrovirus that will be used to cause overexpression of PKC-ζ in intact mice and in cultured B and T lymphocytes, to test this notion.

2. Isoform-specific Roles in Physiology. We have used expression vectors to produce lines of NIH3T3 fibroblasts and 32D promyelocytes that overexpress individual PKC isozymes and observed the effects of the high levels of each PKC, before and after activation of the kinases with phorbol ester. PKC-ε overexpression in NIH3T3 cells caused transformation *in vitro* and tumorigenesis *in vivo*, while overexpression of PKC-δ slowed growth. Neither of these activities required activation by phorbol ester. Wild-type 32D cells do not differentiate in response to phorbol ester, but two of the eight PKC-overexpressing lines, LTR-PKC-α and LTR-PKC-δ, had attained this differentiation ability, a property that is typical of most human myeloid cell lines and of normal mouse and human promyelocytes. These results indicate that these two PKCs can mediate this form of differentiation.

3. PKC Isoforms Translocate to Different Intracellular Organelles upon Activation by Phorbol Esters. We have used fluorescent antibodies that are specific for individual PKC isoforms to determine the intracellular localization for each member of the PKC family in NIH3T3 cells, before and after activation by phorbol ester. Wild-type NIH3T3 cells contained only PKC-α in any significant amounts, thus we were able to observe the effects of overexpression of all the other isozymes against a constant background. Generally, in the untreated overexpressers the PKC protein was diffusely scattered throughout the cytoplasm, consistent with Western blot data that showed the PKC to be free in the cytoplasm. After activation by phorbol ester, a rapid and isoform-specific translocation to specific intracellular organelles could be observed. Depending on the isoform that was abundant in the cells, we could trace a relocation of PKCs to actin-rich microfilaments, endoplasmic reticulum, Golgi apparatus, nuclear membrane, and cytoplasmic membrane, in some cases concentrating particularly at intracellular contact points. These data indicate that individual isoenzymes have their specific substrates at different subcellular locations. It appears that the carboxyl-terminal ends determine the site to which each PKC isozyme translocates. Overexpression of chimeric PKCs in which portions of PKC-δ and -ε are swapped are underway to test this hypothesis.

IV. Structure and Expression of Mouse Pvt-1 (Huppi)

A chromosomal translocation has been documented in a subset (10-20%) of Burkitt's lymphomas and mouse plasmacytomas which juxtaposes the immunoglobulin light chain κ gene to the Pvt-1 region approximately 260 kb downstream of c-*myc*. The

rapid identification of translocations to Pvt-1 has always posed a formidable problem, since the translocation breakpoints span such a large region (> 200kb). In order to establish a better assay for such chromosomal abnormalities, we have designed an RT-PCR-based method using appropriate Pvt-1 and Cκ oligonucleotide primers. Since this Pvt-1/Cκ chimera is such a common product of the Pvt-1 translocation, we have also made constructs containing Pvt-1/Cκ for studies in transgenic mice (in collaboration with F. Melchers, Basel Inst.). In addition, the Pvt-1/Cκ chimera has been placed in a retrovirus that is designed to be expressed only in mouse B cells. It is anticipated that these constructs may generate B-cell tumors and provide the basis for studies to determine what role Pvt-1 plays in neoplasia and, possibly, in normal B-cell development.

V. Somatic Mutations in Tumors. (Huppi)

1. p53-associated mutations. Mutation of the p53 gene is the most common genetic lesion observed in human cancers. In studies of p53 in experimental mouse models of neoplasia, we have identified an unusual somatic mutation in exon 5 of p53 that apparently contributes to the highly malignant phenotype of a series of adenoviral- or SV40-transformed mouse embryo cells (in collaboration with Y. Eyler & A. Lewis, NIAID). In a related experiment (in collaboration with M. Bergel & J. Hochman, Jerusalem), we have examined p53 in a highly malignant T-cell lymphoma that has been adapted to culture. In this case, malignant and non-malignant phenotypes correlate with the presence of a mutation at residue 246 or residue 242, respectively.

The mouse plasmacytoma is unusual in that only 4.4% (2/45) of the tumors exhibit a mutation of p53. In contrast, (in collaboration with K. Bhatia, I. Magrath, NCI) we have found that nearly 70% of Burkitt's lymphomas present with a mutation of p53. In a follow-up study, we have found a significant correlation between poor prognosis (i.e., mortality) and presence of a p53 mutation in BL patients. We have focused on the plasmacytoma as a unique model for the study of alternative mechanisms of inactivation of the p53 pathway. One candidate, Mdm-2 is known to form stable complexes with p53. We have examined Mdm-2 expression in plasmacytomas but found essentially no changes. More recently, expression of the cell cycle-associated gene Waf-1 (or p21) gene has been found to be activated by wild-type p53. Thus, p21 is another candidate in our search for alternative lesions in the p53 pathway that may be involved in plasmacytomagenesis. Yet another candidate, p16, like p21, suppresses cell proliferation by binding to a group of cell cycle-related molecules, cyclin-dependent kinases or cdk's. We have cloned, mapped and sequenced the genes for mouse p21 and p16 in an attempt to establish an assay for mutations or altered expression of these anti-oncogenes in mouse tumors (in collaboration with D. Givol, NCI). We have designed PCR primers from two highly conserved regions to search for p21 and p16 mutations in mouse plasmacytomas and other mouse tumors.

2. c-Myc mutations. We have identified somatic mutations that cluster in the region of exon 2 in Burkitt's lymphoma and mouse plasmacytoma (in collaboration with K. Bhatia, I. Magrath, NCI). The high frequency of mutation (30-75%), the high ratio of replacement to silent substitutions, and the exclusive presence of the mutations on the expressed allele argues strongly for a selective advantage to these mutations. Interestingly, the region associated with a high incidence of c-Myc mutation (the transactivation domain of exon 2) has recently been identified as the binding site for the retinoblastoma-like gene p107. In collaboration with P. Hamel, Toronto we have initiated a study to determine 1) whether binding of p107 to this region of c-Myc is ablated in tumors that contain c-Myc mutations, 2) whether mutations of p107 can be found in mouse plasmacytomas and 3) whether the

transactivation of c-myc is perturbed. We have cloned, sequenced and mapped the mouse gene for p107 in an attempt to establish primer sequences in the mouse and to generate mouse constructs for study of p107-c-Myc binding. No mutations of p107 have been found to date in the mouse, and preliminary studies indicate that p107 binding to c-Myc is not prevented by mutations of c-Myc. Studies are now focusing on the effect that p107 may have on transactivation of c-Myc.

VI. Gene-specific DNA Repair Deficiencies in Plasmacytoma-susceptible Mouse Strains. (Mushinski, Bohr, Owens, Beecham)

Although the entirety of an organism's genomic DNA can be damaged, repair of damage is not uniform throughout the genome. We have reported that the rate of excision repair of UV-induced DNA damage varies not only among different genes and gene segments but also between inbred strains of mice that differ dramatically in their sensitivity to plasmacytoma formation following intraperitoneal administration of mineral oils. Specifically, the sites at which critical and characteristic chromosomal translocations occur in plasmacytomas are rapidly repaired in lipopolysaccharide (LPS)-stimulated B lymphoblasts from DBA/2 mice (resistant to plasmacytomas) while these same regions are poorly repaired in cells from BALB/c mice (sensitive to plasmacytoma formation). These sites include the 5' flank of c-*myc*, Pvt-1, the IgA heavy chain and the Igκ-light chain genes. Unlike the aforementioned genes, BALB/c lymphoblasts are able to repair the DHFR and c-*abl* genes at rates similar to those found in DBA/2 lymphoblasts. The repair differences between BALB/c and DBA/2 cells could be due to factors that control the accessibility of individual genes to repair enzymes.

RNA transcription is also dependent on accessibility of specific gene regions to enzymes, and it is currently well accepted that actively transcribed sequences are repaired more efficiently than non-transcribed sequences, i.e., there is a direct coupling between repair and transcription. However, we have shown that the steady-state levels of c-*myc* and Pvt-1 mRNAs are the same in DBA/2 and BALB/c lymphoblasts, suggesting that in this situation repair may not be coupled to transcription. It is possible that there is a significant strain difference in transcription at these sites, which might not be reflected in the steady-state mRNA level. Thus we analyzed nuclear run-on transcripts in LPS-induced B lymphoblasts from BALB/c and DBA/2 mice from both DNA strands in c-*myc* and in Pvt-1 and found no significant strain differences. These findings conclusively demonstrate that the interstrain repair difference is not due to difference in accessibility, but, instead, it represents a unique form of gene-specific repair that is not coupled to transcription. It is tempting to speculate that this novel type of repair, or a heritable, defective form of it, might play a role in producing mutational hot spots, i.e., poorly repaired regions in certain specific genes in some familial forms of neoplasia.

VII. Cyclins: (Hanley-Hyde, Mushinski)

Regulation of expression of cyclin D2. Genomic fragments that contain most of the coding region of cyclin D2 were isolated from a genomic library of BALB/c liver, and most of the putative first exon and nearly 5000 bases of upstream flanking sequence were sequenced. This flanking sequence contains 4 c-Myc protein-binding sites that were subsequently shown by mobility shift assays to bind c-Myc and Max proteins. Fragments of the 5' flank were subcloned into luciferase reporter gene constructs and found to direct expression of luciferase efficiently. This suggested that expression of cyclin D2 would be upregulated by c-myc protein, consistent with our observations that B-cell

lines that express large amounts of c-myc protein also contain large amounts of cyclin D2 mRNA. This concept is now being tested.

Genetic Instability. It was observed that transformed cell lines, including plasmacytomas, have genomic abnormalities that include amplifications. In collaboration with S. Mai, Basel Inst.) we have shown that cell lines that express high levels of c-Myc protein, due to chromosome translocation or a variety of other reasons, commonly displayed amplification in a limited number of genes. The amplification was non-random, and the subset of genes most commonly amplified includes cyclins D1, D2, and E, as well as genes for enzymes that are required for dNTP synthesis and replication: dihydrofolate reductase (DHFR); carbamoyl-phosphate synthase/aspartate transcarbamylase/-dihydroorotase (CAD); ribonucleotide reductase, M2 subunit; and, sometimes, the c-*myc* gene itself; but not cyclin C, serum albumin, or the M1 subunit of ribonucleotide reductase. The amplifications were detected Southern blots and also by *in situ* hybridization, and they were not limited to a single tissue type or animal species. Studies are underway to understand how c-*myc* overexpression is linked to this form of genetic instability.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08950-12 LG

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6	3	3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. We are beginning to define fundamental principles that will allow prediction of function from structure, principles that are critical to such applications as antibody and vaccine design. We are investigating 3 important interrelated applications of this paradigm: (1) Molecular basis of immunological recognition: The X-ray structures of HyHEL-5 Fab complexed with 2 single site-directed mutants of HEL have been solved and refined and a third is in progress: R68K which reduces the affinity of the complex by a factor of over 10 to the 3rd power, R68A which reduces affinity to almost nonmeasurable, and R45K, which reduces affinity by only 10-fold. Thermodynamic and kinetic measurements indicate complex formation of these high affinity mAbs is enthalpically driven, and site-directed mutations which decrease affinity also decrease the enthalpic change. (2) Cognate antigen presentation by B cells: Experiments with B-cell lymphomas expressing the HyHEL-10 sIg from HyHEL-10 transgenic mice have demonstrated that B-cells can present HEL recognized by their surface Ig up to 4 orders of magnitude more effectively than unrecognized antigen. Depending upon the specificity, anti-HEL antibodies can differentially affect presentation of HEL to T-cells. (3) Antigen delivery systems for vaccine development: A recombinant B19 parvovirus particle has been used to present recombinant HEL to the immune system. The handling of this antigen in B-T cognate interactions is being investigated. We are also investigating immunogenicity and protective epitopes in Shigella flexnerii. We have initiated a new breast cancer project. Two division-competent epithelial cell populations have been identified which are candidate stem-cell and progenitor cells. Their roles in normal and neoplastic development of mammary glands are being studied.

Major Findings

We have continued to place major emphasis on investigating molecular recognition. The principal focus 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). With the X-ray structures of 3 Fab-HEL complexes available, we are experimentally investigating structure-function relationships in the interface by manipulation through site-specific mutagenesis of both antigen and antibodies. These studies are beginning to define fundamental principles that are critical to such applications as antibody design and vaccine development, and have direct applicability to structural aspects of T-B cognate interactions underlying regulation of immune recognition and development of the immune response. The molecular recognition results also provide a model for studies utilizing mAbs to probe structure-function relationships of other biologically important regulatory proteins whose structure and function are less well defined. We anticipate that these principles will be applicable to future studies on regulatory molecules important in mammary gland stem cell function, a new project which we are initiating.

A. Molecular and Biological Basis of Immunological Recognition

1. The molecular basis of antibody-antigen complementarity - Dr. Smith-Gill, Mr. M.A. Newman, Dr. J.-C. Grivel

Among late-response anti-HEL antibodies there appear to be contrasting structural solutions 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 V_H36-60 and Vκ23 gene families and epitope mapping experiments suggest identical epitopes are recognized. Low resolution x-ray structures of HyHEL-26 and HyHEL-8 complexed with HEL have recently been solved, and confirm that the epitopes recognized by these mAbs are nearly identical to that of HyHEL-10 (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 V_L-V_H 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 this epitope are limited. This hypothesis predicts that other mAbs functionally similar to HyHEL-10 will also utilize members of the V_H36-60 and Vκ23 gene families. To date, one of these, HyHEL-32, has been shown to utilize V_H36-60. The recent solution by Dr. E. Padlan of the x-ray structure of the uncomplexed Fab HyHEL-10 will provide important data pertinent to the question of whether complex formation is accompanied by conformational changes.

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 HyHEL-5, but may not be as closely coincident as those recognized

by the HyHEL-10 series. At least 2 heavy chain V_H families, V_H36-60 and V_HJ558, are expressed by these functionally related mAbs. The HyHEL-501 V_H is similar to HyHEL-5 in V_HJ558 gene usage and identical CDR3 length, but the sequences of their joining regions differ considerably. The HyHEL-503 heavy chain is similar in gene usage and CDR3 length to the anti-HEL mAb HyHEL-10, even though these antibodies bind in different areas of HEL.

Mr. Newman is developing a system for site-directed mutagenesis and prokaryotic expression of the antibody Fab. We have received plasmid vectors from Dr. Richard Willson at the (U Houston) with whom we are collaborating on the expression of HyHEL-5 and HyHEL-10 in *E. coli*. This vector will allow the insertion of immunoglobulin V-region region sequences into a reading frame for expression in *E. coli*. Each intermediate vector contains constant region sequences for either immunoglobulin heavy or light chain, restriction enzyme sites for insertion of variable region DNA, and the appropriate signal sequences for induction of expresssion and transport of product out of the cytoplasm. Mr. Newman has constructed intermediate plasmids in vitro that contain the V_H and Vκ regions of HyHEL-5, and transformed *E. coli* JM101 host cells. The V_H and Vκ regions of HyHEL-10 have been expressed in this vector, and we are working on optimizing conditions for obtaining usable quantities of HyHEL-10 protein.

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 and Dr. R. Willson, U Texas). 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 at least 2 orders of magnitude, and which will be the first published structure of a mutated antigen in complex with an antibody generated to the unmutated, native antigen (Chacko *et al.*, in press). 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, and crystallization trials are in progress with the site-directed mutant R68A. The new BiaCore BioSensor recently installed in our laboratory will allow us to obtain kinetic data on these and other site-directed mutants, thus allowing us a new level of precision in analysis of the functional correlates of structural changes.

Definition of the structures of Fab-HEL complexes by X-ray crystallography has made them attractive systems for thermodynamic studies. Microcalorimetric data from the laboratory of R. Willson for the complex of HEL with HyHEL-5 showed that the association of both antibodies with HEL was enthalpically driven (Hibbits *et al.*, 1994, *Biochem.*) **33**:3584). Isothermotitration calorimetry measurements of ΔH and ΔCp for the HyHEL-5/HEL association were made under a variety of temperature and pH condiditons. The values of ΔH and ΔCp were comparable to those of other protein-protein complexes. ΔH was found to be large and negative, and decreased linearly with temperature, and was independent of pH and buffer ionization enthalpy. The association was distinguished by a negative entropy at all temperatures studied and the extrapolated temperature at which ΔS becomes positive is below 0°C which is unusual among protein-protein systems. We are collaborating with Dr. Willson's laboratory

perform kinetic and thermodynamic experiments on the complexes of HyHEL-5 with mutant forms of HEL, and on complexes of HyHEL-10. HyHEL-10 complex formation affinity for either mAb also decrease the enthalpic loss, but in the case of Hy-HEL-5 there is not a good correlation between affinity and ΔH.

The negative entropy change associated with HyHEL-5-HEL complex formation contrasts with haptens, which undergo a increased entropy change upon complexing with antibody. Although Bhat *et al.* (1994, *Proc. Natl. Acad. Sci.* USA, **91**, 1089) concluded that that hydration enthaply should be an important factor in complex stabilization of D1.3-HEL complex formation, our results suggest a different interpretation of thermodynamic data. Our mAbs HyHEL5 and HyHEL-10 each have higher affinities (by several orders of magnitude) for HEL than does D1.3, and the complexes of Fabs HyHEL-5 and HyHEL-10 with HEL appear to have fewer water molecules in the interface than does that of D1.3. In addition, the affinity of HyHEL-5 for lysozyme with an R68K substitution is over 2 orders of magnitude lower than it is for HEL. Comparisons of Fab HyHEL-5 complexed with native HEL and the site-directed mutant R68K of HEL suggest that the hydrogen bridging by water in the interface does not completely compensate for incomplete complementarity of the antigen and antibody. $Arg68_{HEL}$ in the native structure forms 2 salt bridges to $Glu50_{VH}$ which are major contributors of binding energy and the hydrogen bonding capacaties of this residue are fully utilized. In contrast, $Lys68_{HEL}$ in the mutant complex has fewer hydrogen bonds and makes no salt links; its hydrogen bonding capacities are not fully utilized, despite the retention of a new antigen-bound water in the interface which replaces the "hole" created by reducing the side chain volume of residue 68. There is little difference between the buried surface areas in the native and mutant complexes, considering the new water associated with the $Lys68_{HEL}$ as part of the HEL buried surface. In this case, the specific protein-protein interactions appear to account for the differences in affinity of the native and mutant complexes. While overall, buried surface areas do not appear to account for affinity differences among various antibody-protein complexes, it may be that the degree of complementarity, as indicated by the presence or absence of water molecules in the interface, may be a better indicator of high affinity interactions. In other words, water molecules do not necessarily constitute a functional "paste" which creates complementarity, but rather they appear to compensate for lack of perfect complementarity which would allow the highest affinity direct protein-protein interactions. In fact, preliminary analysis by surface plasmon resonance of the kinetics of HyHEL-5 complex formation with HEL and with bobwhite quail lysozyme, which contains the Arg68Lys mutation, indicates that both the on and the off rates of the mutant lysozyme are higher. Thus, the less prefect complementarity creates a complex that is less stable, despite the inclusion of a water molecule. Microcalimetric experiments in progress will allow a partial testing of this hypothesis.

2. Cognate antigen presentation by B cells - Dr J.-C. Grivel

Among antigen presenting cells, B cells play a special role. They have the ability to present the antigen recognized by their sIg (surface Immunoglobulin), 10^3-10^4 times more effectively than unrecognized antigen. This enhanced antigen presentation for the cognate antigen is the result of the sIg mediated antigen internalization. However, when compared to their ability to be

stimulated by antigen specific B cells, some T cells are efficiently stimulated whereas others are not at all, showing that depending on the fine specificity of both B and T cells, B cells can either enhance or impair presentation of their cognate antigen. This differential antigen presentation by B cells is the result of the intimate interaction of the antigen and the sIg which, by altering the processing of the antigen, modifies the generation of certain T cell epitopes. This mechanism has been described in some experimental models. However, the poor information about the interaction of the studied antigen and the sIg expressed by the B used, as well as the lack of a panel of T cells whose antigenic specificity cover the whole antigen, limited considerably previous studies.

Dr. Grivel has developed mice transgenic for the genes encoding a μ-δ form of the anti-HEL antibody HyHEL-10. Founder pairs from these transgenic mice, backcrossed 10 generations onto a C57B6 background, are currently in quarantine at FCRDC; we plan to cross the transgenes onto the BALB/cAnPt background in our FCRDC colony. B cells from these transgenic animals are exquisitely efficient in presenting HEL to HEL specific T cell hybridomas, requiring in general 10^3-10^4 (10^{-2} nm vs. 10-100 nM HEL) times less antigen than normal B cells to present HEL to T cells. We have derived B x B cell lymphomas by fusing these transgenic B cells with B cell lymphomas. Depending on the MHC haplotypes of the B cells and the lymphomas used, these hybrids express different MHC encoded molecules together or not with the anti-HEL sIg receptor. We have obtained series of T cell hybrids specific for HEL peptides in different MHC contexts from Drs. L. Adorini (Roche Milano Riccerche) and N. Shastri (U. Cal., Berkeley) with whom we are collaborating on this project. The specificity of these hybrids covers almost the entire HEL sequence. In order to obviate any effect of the interaction of HEL with the sIg in antigen processing, we will compare the ability of transgene positive and negative lymphomas to present HEL to this panel of anti-HEL T cells. Moreover, we will extend this study to the investigation of the effect of other anti-HEL antibodies in the presentation of HEL to T cells. This will be achieved by the use of anti-HEL antibodies belonging to complementation groups different from that of HyHEL[10]; we have antibodies covering most of the HEL surface. Knowing both the B and T cell epitopes, this study will allow a better dissection and understanding of antigen processing and presentation by B cells.

Preliminary results on a T cell line specific for the peptide 46-61 of HEL shows that significant T cell stimulation is measurable at HEL concentrations as low as 10^{-11} M. Anti-HEL antibodies thought to have very close epitopes on HEL, affect differently the presentation of this determinant to T cells. Application of this approach to the 15 remaining T cell lines will be very informative.

In a second aspect of our work, we are focusing on antigen presentation function of B cells in vivo. In addition to the engagement of their receptor, T cells need to receive co-stimulatory signals in order to be activated. In the absence of co-stimulation, recognition of antigen by T cells, instead of inducing a T cell response, anergizes the antigen-specific T cells. For these reasons, the activation status of B cells is paramount in their ability to activate T cells. Small resting B cells which don't express co-stimulatory activity induce T cell anergy when presenting antigen to T cells. However, cross-linking of B cells' sIg by the antigen, induces the expression

of co-stimulatory molecules, allowing these B cells to activate T cells. The requirement of receptor cross-linking for T cell activation by B cells raises the question of the relevance of antigen presentation by B cells in vivo. Which kind of antigen is presented by B cells in vivo? Obviously, soluble proteins are not relevant candidates for the induction of B cells' sIg cross-linking, the most likely antigens are particulate antigens which can express multiple copies of a single antigenic determinant on their surface. These antigens are not classically considered as B cell antigens since, due to their poor phagocytic capacity, B cells have not been shown to be able to present particulate antigen. Withstanding these observations, there is some indirect evidence of the involvement of B cells in the presentation of viral particles to T cells in vivo. Moreover, we have been able to demonstrate that B cells can present liposomes-coupled antigens to T cells in vitro when these liposomes are of a size compatible with receptor mediated endocytosis (< 200nm). Interestingly, in one antigenic model, receptor mediated antigen presentation by B cells was shown to give rise both to Class I and Class II restricted presentation, giving to B cells the ability to stimulate both helper and cytotoxic T cells. Our hypothesis is that viral particles, which fulfill both the size and the receptor cross-linking criterion, are the physiologically relevant antigen presented by B cells in vivo. Moreover, the ability of B cells to present antigen both in the Class I and Class II MHC context, gives them a central role in the initiation of anti-viral immune responses. We are investigating these possibilities in our antigenic model.

According to the current knowledge of antigen presentation by B cells *in vivo* and to our hypothesis, different physical forms of HEL will give rise to different anti-HEL responses at the T cell level after injection in mice. Soluble HEL will induce a T cell anergy. On the other hand, multiple HEL molecules expressed on the surface of a particulate antigen will induce a T cell response if the particles are of a size compatible with receptor mediated endocytosis. We already have, through a collaboration with N. Young (NIHCB), parvovirus capsids expressing HEL on their surface (see Sec. 3a below). These capsids, soluble HEL, and bacterium transfected with HEL-encoding plasmids, will be used in the investigation of antigen presentation by B cells in vivo. Our hypothesis predicts that only HEL-expressing parvovirus capsids will induce an anti-HEL T cell response upon injection in mice reconstituted with transgenic B cells expressing the anti-HEL antibody HyHEL-10. Soluble HEL will induce a T cell anergy and bacterium expressing HEL will only be presented by macrophages allowing the comparison of macrophage-induced T cell responses and B cell-induced T cell responses.

3. Antigen delivery Systems for Vaccine Development

   a. Parvovirus particles as platforms for protein presentation - Dr. Smith-Gill, Dr. Grivel, in collaboration with Dr. Neal S. Young, Hematology Branch, NIHLB

The B19 parvovirus is a human pathogen which infects erythroid progenitor cells of bone marrow, blood, and fetal liver. The capsid contains two structural proteins. The major capsid protein VP2 comprises over 95% of the capsid protein. The minor capsid protein, VP1, is not required for capsid formation, and differs from VP2 in the addition of a unique region of 227 amino acids at the amino terminus, at least some portion of which is external to the capsid. The

predominant antigenic specificity in human convalescent sera is directed to VP1, not VP2; anti-VP1 can neutralize virus. Previous experiments from Dr. Young's laboratory have established that the VP1 unique region and the VP1-VP2 junction contains multiple linear neutralizing epitopes, and that empty capsids formed by VP2 alone are equivalent to natural capsids by morphologic criteria.

In order to determine whether the unique region of VP1 could be substituted with another protein sequence for the purpose of antigen delivery, empty capsids of B19 were expressed in a baculovirus sytem, and the gene encoding HEL was substituted for the unique region of VP1 with variable amounts of retained VP1 sequence (Miyamura et al., in press). All purified recombinant capsids contained HEL. The external presentation of HEL was demonstrated by immunoprecipitation, ELISA, and immunoelectronmicroscopy using anti-HEL mlAbs or specific rabbit anti-HEL. That expressed HEL was properly folded was demonstrated by showing the ability of empty recombinant capsids lyse *Micrococus lysydeikticus* cell walls, and reactivity with mAbs HyHEL-5, HyHEL-10, and HyHEL-15, known to be specific for the native conformation. Preliminary expriments indicated that rabbits inoculated with recombinant capsids produced low titer antisera reactive to HEL. Future experiments will be focused on increasing the proportion of recombinant VP1 in the capsids, in order to increase the antigenic valency, and on analysis of the murine B and T cell responses to the recombinant capsids. This system has a high potential for targetted gene therapy as well as immune system stimulation.

b. Nature of the protective response to *Shigella* - Mr. Mainhart, Dr. Smith-Gill, 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.

Mice were immunized with *Shigella flexerni* 2a by using various conjunctival infection protocols. Monoclonal antibodies were obtained by fusing spleen cells from these mice with SP2/0 cells. Four monoclonal celllines were isolated that produce immunoglobulins with a relatively high binding specificity for the LPS 2a serotype. Three of these anti-LPS antibodies had the IgM isotype and the forth was an IgG. These mAbs agglutinate live *Shigella*. These results demonstrate that monoclonal antibodies recognizing unique LPS serotypes can be derived from mice immunized by mucosal routes.

The heavy and light chain sequences of the above anti-LPS immunoglobulins are now being determined using PCR cloning and dideoxy nucleotide sequencing protocols. The amplified genes of one IgM monoclonal antibody have been completely determined. Its light chain variable

region gene belongs to the Vκ23 family whereas that of its heavy chain is a rare V_H sequence that has not yet been assigned a family, but it is expressed in some anti-phenyloxazalone mAbs. Once all the above anti-LPS mAbs have been sequenced, their variable regions will be placed into a bacterial expression system to examine the effects of structural changes on antigen binding.

B. Epithelial Stem Cell Function in Normal and Neoplastic Mammary Gland Development - Dr.Chepko, in collaboration with Dr. B. Vonderhaar and Dr. G. Smith, LTIB, DCBDC.

This represents a new direction of research for our group. The purpose of this research is to identify a potential stem cell population in mammary gland epithelium, and to determine its function and the factors that regulate it in normal and neoplastic mammary gland development. Dr. Chepko has identified two division competent cell types in rat and mouse mammary gland epithelium. These two cell types are potential candidates for stem cells. The specific focus of current research is: 1) to immunophenotype these two cell types in mouse mammary epithelium with regard to receptors, 2) to describe their population kinetics in normal and neoplastic mouse mammary gland and 3) to compare via in situ hybridization the active genes in each cell type in hormone dependent and hormone independent neoplasias.

In collaboration with Dr. G. Smith, Dr. Chepko has found evidence for two division competent cell types in rat and mouse mammary gland. These two cells stain very lightly with a variety of stains and have been called clear cells. They differ from each other in size and are thus informally designated: large clear cells and small clear cells.The large clear cell, already described by several authors in the literature, is at present a candidate for a mammary epithelial stem cell. However, in the mouse this cell, although it undergoes mitosis without synthesizing DNA (Smith and Medina, 1988, *J. Cell Science* **89**: 173) divides and synthesizes casein and K6 cytokeratin, does not take up tritiated thymidine it is no longer present in rat or mouse epithelium after the sixth day of lactation. This behavior is consistant with the function of a progenitor cell but not with a stem cell. The other division competent cell (the small clear cell) in rat and mouse mammary epithelium remains cytoplasmically undifferentiated in every stage of the rat reproductive cycle. Both cell types were followed through a complete reproductive cycle in rat and examined in mammary explants from pregnant mice to observe their structure in different functional stages of the mammary gland. Both cells divide during mid-pregnancy and at parturition in the rat and the large clear cells increase in number in mouse explants cultured in medium containing insulin, hydrocortisone and prolactin. Whereas the role of the cell which contains secretory product is clear: it gives rise to more cells like itself and synthesizes milk proteins; that of the role of the small clear cell is not clear. At this time we only know: 1) that it undergoes division at periods of peak mitosis, 2) it appears as a singlet, in pairs with itself or with the large clear cell, 3) it comprises an average of 2-3% of the mammary epithelial cells in rat, 4) it does not contain secretory organelles or reach the lumen at any time, 5) it does not undergo post-lactational autophagocytosis as do all the other cell types in the epithelium, 6) they are consistantly present in all functional stages of rat and mouse mammary gland including

virgin and 6) it looks so much like a lymphocyte that some investigators have proposed it to be part of a mucosal immune system for mammary epithelium. Our working hypothesis is that the small clear cells represent stem cells, which give rise to daughter cells that develop into large, clear progenitor cells. These results are currently being prepared for publication.

The above information and observations made by Dr. Barbara Vonderhaar with her *in vitro* whole organ culture of mammary gland are the basis of a collaboration project with Dr. Vonderhaar and her laboratory to label newly synthesized DNA with BdUr in mammary gland of mice primed with estrogen and progesterone to determine 1) at which points in the mammary gland tree cell divisions take place and 2) which cell types participate in DNA synthesis and/or mitosis. Dr. Chepko has also begun a pilot study using immunohistochemistry to screen antibodies to markers such as stem cell factor, estrogen and progesterone receptors, T and B lymphocyte markers and various oncogene proteins so that a study can be designed and executed to determine which particular cell types are susceptible to carcinogens under which set of hormonal conditions. Dr. Vonderhaar's whole organ culture system will be utilized in this study because the hormones can be manipulated to cause epithelial growth, lactation, involution and regrowth. With this system we hope to be able to differentiate the roles of the two division competent cells and describe the normal cell kinetics of the mammary gland as well as the processes involved in neoplastic transformation at the cellular level.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08952-08 LG

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Role of the c-myb in myeloid tumorigenesis

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

P.I.:	L. Wolff	Senior Investigator	LG, NCI
	V. Nazarov	Visiting Fellow	LG, NCI
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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, Univ. of California, Irvine, CA; B. Hoffman, Fels Institute, Philadelphia, PA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Our laboratory has conducted research in two general areas: the first area involves investigations on susceptibility and resistance to MuLV induced myeloid leukemia (MML), and the second area involves investigations on the effects of c-*myb* on myeloid differentiation in vitro. Our previous work had shown that although adult BALB/c and DBA/2 mice are susceptible to MML, 7 other strains are resistant. In addition, resistant NFS and C3H/He mice were demonstrated to support replication of the inducing virus, M-MuLV and support c-*myb* activation in hematopoietic tissues. It appeared that early stages of leukemia were permitted, but preneoplastic cells were unable to progress. Recent evidence indicates that a T-cell mediated immune response involving non H-2 gene(s) is responsible for eliminating preleukemic cells in resistant mice. Interesting, resistance to F-MuLV induced MML in BALB/c mice was also shown to be in part related to immunosurveillance phenomena since F-MuLV was shown to induce MML in immunocompromised BALB/c *nu/nu* (nude) mice. This study led to the surprising finding that in the leukemias induced by F-MuLV in BALB/c nude mice, activation of c-*myb* by the typical mechanism involving 5′ integration and formation of *gag-myb* RNA was not operative. However, in some leukemias new unique sites of proviral integration were found at the distal 3′ end of gene within the last intron. Our new in vitro studies on differentiation were aimed at helping us to determine how myb transforms cells. This has led us to the conclusion that ectopic expression of *myb* does not block the early maturation of neutrophilic granulocyte differentiation. However, when full length Myb, or tumor-specific truncated forms of Myb, are overexpressed, they prevent the late nonmitotic phases of the pathway.

Major Findings

I. Acute myeloid leukemia model

Over the past several years the laboratory has been studying a mouse model for acute myeloid leukemia (AML). The leukemic cells have been classified as promonocytes on the basis of their morphological and functional characteristics and therefore, more specifically resemble human acute monocytic leukemia (AMoL) (M5). The induction of leukemias in our animal system requires both the intravenous inoculation of replication competent murine leukemia virus (MuLV) and intraperitoneal inoculation of pristane. It has been shown that the role of the retrovirus in the disease is to mutagenize a proto-oncogene(s) through the mechanism of proviral insertion (insertional mutagenesis), whereas the role of pristane is to create a chronic inflammatory granuloma that stimulates leukemic cell development and enhances outgrowth in the peritoneal cavity. These neoplasms have been named MML based on the fact that they are MuLV- induced myeloid leukemias.

A major part of our past and present studies has focused on insertional mutagenesis of the proto-oncogene c-*myb*. Although it is probably not the only target of insertional mutagenesis in this model, we have found it to be activated 100% of the time in leukemias induced by Moloney, and activated in 66% and 50% of leukemias induced by Amphotropic 4070 and Friend FB29, respectively. We reported last year that the activation of c-*myb*, defined as the complete process of viral integration, transcription from the viral LTR, and formation through splicing of fusion *gag-myb* RNA, occurs very early after inoculation of BALB/c mice with Moloney virus. Using a sensitive RT-PCR technique designed to detect *gag-myb* mRNA at the level of 1 cell out of 10^6, it was found that cells with activated *myb* could be found in the bone marrow and spleen within the first month after infection and at later times (two months) in the peritoneal granuloma tissue. These studies suggest that the target for transformation is a progenitor cell and that c-*myb* activation is the first step in the leukemogenic process. Furthermore, it suggests that as the leukemia develops three events are necessary, differentiation to a mature phenotype, further transformation and migration to a new site.

Factors affecting susceptibility and resistence of mice to MML. Recently we began a study aimed at elucidating mechanism(s) of resistence to MML in certain strains of mice. Although BALB/c and DBA/2 mice are both susceptible to disease, 7 other strains that were tested were resistant. Using F1 hybrids between BALB/c and either C57BL/6, NFS, or C3H/He it was demonstrated that resistence is dominant. The following potential mechanisms of resistence to the virus-induced leukemia were proposed (1) block to virus infectivity; (2) inhibition of virus integration into the c-*myb* locus or formation of leukemia-specific *gag-myb* mRNAs which could be tested using the sensitive RT-PCR approach for aberrant mRNAs; (3) immune response to virus and/or preleukemic cells; (4) inability of preleukemic cells to undergo a secondary oncogenic event; (5) insufficient environment in peritoneal cavity for leukemic cell outgrowth. Last year we reported results of studies that looked at the first two mechanisms in three resistant strains of mice, C57BL/6, NFS, and C3H/He. Briefly, it was

found that C57BL/6 mice were unable to support early virus replication. However, NFS and C3H/He mice supported replication and were shown to have cells in both hematopoietic organs that expressed *gag-myb* mRNA. Because it was noted, however, that the level of virus replication in these mice was an order of magnitude lower than that in BALB/c mice and the number of mice positive for the RT-PCR products was lower than that in the susceptible strain, we considered the possibility that the NFS and C3H/He mice might have a stronger immunological response to the virus or virus-infected cells compared to BALB/c mice. To test this possibility we performed the induction experiments using Moloney (M)-MuLV and pristane in immunocompromised C3H/He *nu/nu* mice and C3H/He sublethally irradiated mice. Interestingly, 7 out of 20 inoculated C3H/He *nu/nu* mice and 4 out of 20 to date of inoculated C3H/He irradiated mice developed leukemias of the promonocytic phenotype observed in BALB/c mice. In order to confirm that the derived tumors had the activation of *c-myb* characteristic of Moloney MuLV-induced tumors in susceptible strains, we analyzed DNA for rearrangements of the 5' end of the c-*myb* locus and RNA for the expression of *gag-myb*. Five out of five leukemia-derived DNAs tested were positive for 5' *myb* rearrangements and also expressed high levels of aberrant fusion RNA. It was concluded from these studies that, at least in the case of C3H/He mice, resistence is not due to an inability of the virus to activate c-*myb* or to a lack of other tumor potentiating events. Furthermore, it was concluded that the pristane environment in C3H/He mice is sufficient for enhancement of tumor outgrowth. Rather it is hypothesized that leukemia development is restricted in C3H/He by an immune response, presumably T-cell mediated. Further experiments, using mice congenic at the major histocompatibility locus demonstrated that nonH-2 genes are required for resistence in both C57BL/6 and C3H/He mice. This animal model for AML is a unique system for investigating tumor progression as it relates to the immune response.

Further studies on differences in ability of Friend and Moloney MuLV to induce MML and their role in c-*myb* activation. Although M-MuLV is capable of inducing promonocytic leukemia in 50% of BALB/c mice injected with pristane, Friend MuLV strain 57 (F-MuLV) is nonleukemogenic under similar conditions. Previous results using reciprocal recombinant viruses showed that two regions, Ψ-*gag*-PR and *env*, are responsible for the inability of this virus to induce MML. We first evaluated whether a lack of viral infectivity or an inability of the virus to activate c-*myb* in the bone marrow and spleen could account for the lack of leukemias in F-MuLV mice. However, infectivity of the bone marrow was shown to be high and there was evidence for expression of *gag-myb* mRNA in hematopoietic tissues. Recently we tested the hypothesis that the lack of F-MuLV leukemias might be due to an increased stimulation of the immune response to virus and/or virus-infected cells, because it was conceivable that differences in the two viruses in *env* and *gag* might result in variations in immune stimulation. In order to address this possibility we looked at the ability of F-MuLV to induce leukemia in pristane-treated immunocomprised mice. BALB/c mice which received irradiation (600 rads) and BALB/c *nu/nu* (nude) mice were inoculated intraperitoneally with pristane and intravenously with F-MuLV. It was found that nude mice were susceptible to F-MuLV induction of promonocytic leukemia with an incidence of 41%, although irradiated mice continued to be resistant to leukemias. An

interesting finding was that leukemias induced in nude mice did not have evidence for c-*myb* activation involving proviral insertion within the 5' end of the gene through the formation of *gag-myb* mRNA, a phemomenon which is invariable in M-MuLV-induce MML. Only one tumor had a rearrangement at the 5'end of the gene, but there was no evidence that *gag-myb* transcripts were formed in this leukemia. We suggest from these experiments that the resistance to F-MuLV induced promonocytic leukemia is in part due to increased immune stimulation of T-cell related mechanisms in these mice and in part due to an inability to activate c-*myb* in the proper cellular environment. Perhaps a cell specific splicing mechanism exists which could account for our observations that *gag-myb* message is formed in hematopoietic tissues in F-MuLV infected mice, but is not observed in the F-MuLV-induced leukemias.

II. Some Friend strain 57 induced promonocytic leukemias in nude BALB/c mice and irradiated DBA/2 mice show new mechanisms of c-*myb* activation

The previously reported mechanism of activation of *myb* by M-MuLV by proviral insertion into the 5' end of the locus and splicing to form *gag-myb* mRNA appears not to play a role in tumor formation in Friend strain 57-infected mice. Although, in immunocompromised nude BALB/c mice and in irradiated DBA/2 mice F-MuLV can induce leukemias with an incidences of 41% and 69%, respectively, there is no evidence that c-*myb* activation occurs by this mechanism. Interesting, some of the tumors have been found by Northern analysis to overexpress small aberrant c-*myb* mRNAs (2.3 - 2.9 kb) despite the fact major rearrangements of the gene locus could not be demonstrated by Southern analysis. In addition, in some leukemias large myb transcripts (5.3 kb) are expressed. In these latter cases we have determined that F-MuLV integration has occurred much farther downstream than observed previously in any MuLV-related leukemias. In order to localize the new integration sites in the F-MuLV induced leukemias, it was necessary to clone the 3' portion of the normal c-*myb* gene from DBA/2 liver DNA and determine the exon-intron structure. From this data we have found that in 2 leukemias proviral integrations have occurred within the last intron of the gene between exon 14 and 15.

III. Effects of ectopic expression of murine c-*myb* and truncated leukemia-specific forms on granulocyte and macrophage differentiation in vitro

Myb has effects on differentiation of myeloid cells that may directly or indirectly correlate with its ability to transform and a major objective in our lab is to determine how c-*myb* blocks differentiation. It has been demonstrated that overexpression of c-myb causes inhibition of differentiation within the erythroid and macrophage pathways. In our first set of experiments we set out to determine if ectopic expression of c-*myb* could block granulocyte differentiation, another arm of the myeloid pathway, and determine if the truncated forms of Myb expressed in MML had the same or different effects on the pathway as full length Myb. Recombinant *myb* retroviruses were used to infect an interleukin-3 (IL-3)-dependent progenitor cell line, 32Dcl3, which undergoes terminal differentiation to mature neutrophilic granulocytes in the presence of granulocyte colony-stimulating factor (G-CSF).

Overexpression of c-*myb* did not abrogate the IL-3 dependency of the parental, however, cells expressing all forms of c-*myb* were blocked at an intermediate stage of G-CSF induced granulocyte differentiation and continued to proliferate in the presence of G-CSF. After 14 days in medium with G-CSF, *myb* expressing cultures predominantly consisted of promyelocytes with some myelocytes and almost undetectable numbers of neutrophilic granulocytes. This suggested that early stages of granulocyte differentiation were not inhibited, a finding that was further supported by the induction of myeloperoxidase, a biochemical marker of promyelocytes. Interestingly, the expression of lactoferrin, known to be a marker of late stages of granulocyte differentiation was completely inhibited in cells infected with *myb* viruses. Our conclusions are that, in cells overexpressing *myb,* granulocyte differentiation program can progress through its initial mitotic phases, but is halted at an intermediate stage coincident with the normal conversion to growth arrest.

We also asked to see if *myb* would have a similar effect on macrophage differentiation (e.g. intermediate block). This was a particularly interesting question because our MML cells have an intermediate morphology and support the prediction that mouse *myb* does not completely block differentiation to macrophages. Studies reported by other laboratories investigating the effect of *myb* ectopic expression on differentiation of progenitor cells to the monocyte/macrophage lineage differed in their results. Although human c-*myb* was shown to completely block M1 cell differentiation at the myeloblast stage, mouse c-*myb* was shown by another group to only partially block differentiation of WEHI-3B cells at the late promonocytic stage. We were interested in determining if the partial block to macrophage differentiation seen in WEHI-3B cells was an effect specific to the mouse gene and could be reproduced in M1 cells. We have begun testing effects of ectopic expression of *myb* on Il-6 induced M1 differentiation in a collaboration with Barbara Hoffman at the Fels Institute. Early results based on morphological analysis, suggest that the cells expressing *myb* are blocked in differentiation at an early stage. These cells also proliferate at a faster rate during the first week in the presence of IL-6 compared to uninfected M1. However, they do not proliferate at the same rate as cells in the absence of IL-6. A northern analysis demonstrates that although ferritin, an intermediate differentiation marker, is induced in *myb* infected cells, lysozyme, a late marker, is not.

Publications

Mukhopadhyaya R, Richardson J, Nazarov V, Corbin A, Koller R, Sitbon M, Wolff L. Different abilities of Friend Murine Leukemia Virus (MuLV) and Moloney MuLV to induce promonocytic leukemia are due to determinants in both Ψ-*gag*-PR and env regions. J Virol 1994;68:in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08953-04 LG

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Mice homozygous for lpr (a defect at the FasR locus) or gld (a defect at the FasL locus) develop autoimmunity and profound lymphadenopathy characterized by the accumulation of two functionally anergic T cell subsets, a predominant B220+ DN T cell population and a minor CD4+B220+ population. Enlarged LN also contain increased numbers of memory CD4+ and CD8+ T cells capable of secreting high levels of proinflammatory cytokines. In the past year we have continued to investigate the origins of the B220+ T cell subsets, the mechanisms leading to the accumulation of memory T cells and the role of the various T cell subsets in the development of autoimmunity. To determine the role of CD8+ T cells in the development of lymphoproliferative disease and autoimmunity, we treated lpr and gld mice chronically with anti-CD8 mAb and also bred lpr and gld mice with inactivated β2 microglobulin genes that do not produce CD8+ T cells selected on MHC class I molecules. Studies with these mice revealed that 1) the majority of B220+ DN T cells are derived from positively selected peripheral CD8+ T cells, 2) CD4+B220+ are a separate lineage derived from selected CD4+ T cells, 3) a small proportion of CD4+B220+ T cells may differentiate into B220+ DN T cells, 4) CD8+ T cells negatively regulate the accumulation of CD4+ T cell subsets and autoreactive B cells, and 5) CD8+ dendritic cells may play a role in the accumulation of CD4+ T cell subsets. Ongoing studies of lpr and gld mice treated with anti-TNF-α, -IFN-γ or Vβ8 mAb showed that the accumulation of B220+ T cells and memory T cells is not dependent on IFN-γ, TNF-α or Vβ8+ T cells but that IFN-γ and TNF-α may influence the production of autoantibodies, On the basis of these studies and others with super Ag-treated mice, we propose that FasR/FasL interactions play a critical role in regulating the numbers of peripheral T cells, especially CD8+ T cells, that survive after an immune response and in preventing the accumulation of autoreactive lymphocytes.

Major Findings

I. T cell abnormalities associated with homozygosity at lpr or gld

Mice homozygous for the non-allelic mutant genes 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 $CD4^-CD8^-B220^+$ ($B220^+$ double negative, DN) T cells. In addition to these cells, enlarged lpr and gld LN and spleen also contain increased numbers of B lymphocytes, $CD4^+$ and $CD8^+$ T cells and another unique population of functionally anergic $CD4^+B220^+$ T cells. A high proportion of the $CD4^+$ and $CD8^+$ T cells in lpr and gld LN have the properties of memory T cells and secrete very high levels of IFN-γ and TNF-α following stimulation. When introduced into mice that develop delayed onset, chronic autoimmune disease, such as MRL/Mp, lpr and gld accelerate and exacerbate disease. MRL-lpr mice uniquely develop polyarteritis, infiltrative dermatitis, rheumatoid arthritis, severe immune complex glomerulonephritis and anti-SM autoantibodies. These mice are widely used as models for SLE, Sjögrens syndrome and rheumatoid arthritis.

Recently, both the lpr and gld genes were cloned and identified. lpr maps to the FasR locus on chromosome 19, and lpr mice have an insertion of a retroviral transposon, ETn, into the second intron of FasR that prevents normal transcription of the gene. The FasR locus encodes a 45 kd cell surface receptor (FasR) belonging to the TNF/NGF receptor family. Subsequently, the gene encoding the ligand for FasR was shown to map to the gld locus on chromosome 1. Fas ligand (FasL) is a type II membrane protein homologous to members of the TNF family. In gld mice FasL has a point mutation in the external domain that results in the production of a non-functional protein.

Crosslinking of FasR with anti-FasR mAb has been shown in some systems to induce apoptotic cell death and in others to induce cell activation. Because FasR is expressed on thymocytes, it has been proposed that FasR/FasL interactions may play an important role in the deletion of self-reactive or bystander T cell precursors in the thymus. However, studies in many laboratories, including ours, have failed to reveal any gross abnormality in thymic negative or positive selection in lpr and gld mice. The vast accumulation of anergic $B220^+$ DN T cells and the increased numbers of primed $CD4^+$ and $CD8^+$ T cells and autoreactive B cells in lpr and gld mice, imply that normal FasR/FasL interactions are vital for maintaining homeostasis in lymphoid organs and for preventing the accumulation of autoreactive B cells.

Our long-term goal is to gain a clearer understanding of how the lack of expression of functional FasR and FasL interferes with T cell differentiation, growth, function and survival and leads to the development of autoimmunity. In the past year we have continued to focus on three specific areas of investigation: 1) the identification of the precursors of the $B220^+$ T cell subsets and the reasons for their progressive accumulation; 2) the mechanisms that prevent $B220^+$ DN T cells from responding to stimuli; and 3) the role of $CD4^+$ and $CD8^+$ T cells and cytokines in the development of lymphoproliferative disease and autoimmunity.

II. Developmental Relationships Among T Cell Subsets

A. Chronic injection of C3H-lpr and C3H-gld mice with anti-CD8 mAb prevents the accumulation of $B220^+$ DN T cells but not autoantibody production

Although it is now well established that $B220^+$ DN T cells have undergone normal negative selection in the thymus and, therefore, are depleted of cells making strong responses to endogenous super-antigens or self peptides, there is still debate about which lineage of T cells these cells are derived from. A number of treatments have been reported to reduce the numbers of $B220^+$ DN T cells in LN and spleen. These included neonatal thymectomy, chronic treatment with mAb specific for CD4, CD45(B220), TCR $V\beta8$ or IgM, or chronic administration of 5-azacytidine, cyclosporin A or Staphylococcal enterotoxin B (SEB). While these studies suggested a role for $V\beta8^+$, $CD4^+$ T cells and B cells in the accumulation of DN T cells, they did not clearly identify the precursors of these cells.

To investigate the contribution of $CD8^+$ T cells to the development of lymphoproliferative disease and autoimmunity, we injected C3H-lpr and -gld mice weekly from 3 wk of age with anti-CD8 mAbs specific for mouse CD8.1 or for human CD8 as a control. This treatment resulted in a profound reduction in both the numbers and proportions of $B220^+$ DN T cells. The total numbers of DN T cells/LN in lpr mice were decreased over 400-fold and the proportion of DN T cells was reduced from 75% to 5%. The numbers of $CD4^+$ and $CD4^+B220^+$ T cells and B cells also were decreased, but the proportions of these cells were increased compared to control Ab-treated mice. In all $CD8^+$ T cell depleted mice, the total numbers of B cells, $CD4^+$ and $CD4^+B220^+$ T cells exceeded the number of DN T cells and accounted for most of the residual lymphadenopathy observed. In contrast, anti-CD8.1 mAb therapy had no detectable effects on the proportions or numbers of $CD4^+CD8^+$ DP, $CD4^+CD8^-$ SP, $CD8^+CD4^-$ SP or $CD4^-CD8^-$ DN thymocyte subsets. In addition, depletion of $CD8^+$ T cells had no significant effect on the levels of circulating IgM or total IgG and did not decrease the titres of anti-thymocyte or anti-ds DNA autoantibodies in serum.

In a separate study, C3H-gld mice were treated with anti-CD8 mAb or control mAb for 12 wk. At 16 wk of age after Ab treatment was stopped, one group of $CD8^+$ T cell-depleted mice was thymectomized and the other sham-thymectomized. By 32 wk of age both the control Ab-treated and sham-thymectomized mice developed comparable lymphadenopathy and the majority of the cells ($\sim$60%) in LN were $B220^+$ DN T cells. The thymectomized group also developed significant lymphadenopathy, but in this group $B220^+$ DN T cells only represented 28% of the cells. The majority of the LN cells in these mice were $CD4^+$ and $CD4^+B220^+$ T cells.

These two studies suggested that $B220^+$ DN T cells may arise from peripheral $CD8^+$ T cells, that $CD4^+$ and $CD4^+B220^+$ T cells accumulate in increased numbers in the absence of $CD8^+$ T cells and that $CD8^+$ T cells and $B220^+$ DN T cells do not contribute to B cell hyperactivity or autoantibody production. The data also implied that treatment with anti-CD8

mAb may deplete $CD8^+$ non-T cells, possibly $CD8^+$ dendritic cells, that may be necessary for maximal accumulation of $CD4^+$ and $CD4^+B220^+$ T cell subsets.

B. β_2m knockout lpr mice that lack $CD8^+$ T cells develop lymphoproliferative disease resulting from the accumulation of $CD4^+$ and $CD4^+B220^+$ T cells rather than $B220^+$ DN T cells

To provide further evidence that $B220^+$ DN T cells are derived from MHC class I-selected $CD8^+$ T cells we crossed C57BL/6 $\beta_2m^{-/-}$ knockout mice with C3H-lpr or C3H-gld mice and produced N2 F3 generation mice that were $\beta_2m^{-/-}$lpr/lpr or $\beta_2m^{-/-}$gld/gld double homozygotes. In the absence of β_2m expression, stable class I molecules are not expressed on the cell surface and positive selection of $CD8^+$ T cells in thymus by interactions between TCR on thymocytes and self peptides presented on class I molecules on APC is prevented. As a result $\beta_2m^{-/-}$ mice have few $CD8^+$ T cells in the periphery.

Both the $\beta_2m^{-/-}$ lpr and $\beta_2m^{-/-}$ gld mice had fewer than 1% $CD8^+$ T cells in LN and spleen and, of these, less than 30% expressed TCR α/β. In contrast to the lpr mice depleted of $CD8^+$ cells by antibody treatment, the $\beta_2m^{-/-}$ lpr mice developed early onset and progressive lymphadenopathy and splenomegaly similar to that observed in control $\beta_2m^{-/-}$ lpr or $\beta_2m^{+/+}$ lpr control mice. Although the levels of lymphadenopathy observed between the $\beta_2m^{-/-}$ and $\beta_2m^{+/+}$ lpr mice were similar, the cellular compositions of their LN and spleen were significantly different. The LN of 17 $\beta_2m^{-/-}$ lpr mice aged 15-18 wk were composed, on average, of 24% $CD4^+$ T cells, 32% $CD4^+B220^+$ T cells, 1% $CD8^+$ T cells, 10% $B220^+$ DN T cells and 23% B cells. In contrast, LN from 11 $\beta_2m^{+/+}$ lpr mice contained, on average, 10% $CD4^+$ T cells, 7% $CD4^+B220^+$ T cells, 7% $CD8^+$ T cells, 50% $B220^+$ DN T cells and 21% B cells. Further characterization of the $CD4^+$ T cell subsets and $B220^+$ DN T cells showed that the $CD4^+B220^-$ T cells in both the $\beta_2m^{-/-}$ and $\beta_2m^{+/+}$ mice had similarly elevated proportions of activated or memory-like cells and that there were no significant phenotypic differences between the $CD4^+B220^+$ and $B220^+$ DN T cells in the two sets of mice. A comparison of $CD4^+$ and $CD4^+B220^+$ T cells from $\beta_2m^{-/-}$ mice showed that these two cell populations have remarkably similar proportions of cells reacting with mAb specific for TCR Vβ2, 5, 6, 7, 8.1/8.2, 9 and 10 and that the proportions of Vβ8.3 cells were consistently higher in the $CD4^+B220^+$ subset. Interestingly, the LN cells from $\beta_2m^{-/-}$ lpr but not $\beta_2m^{+/+}$ lpr mice proliferated spontaneously in culture and without exogenous IL-2. These responses peaked 6-7 days after initiation of the cultures. Finally, examination of sera from $\beta_2m^{-/-}$ lpr mice revealed no significant decrease in hypergammaglobulinemia or the titre of circulating autoantibodies specific for thymocytes or ds DNA.

The very significant decrease in the proportions and numbers of $B220^+$ DN T cells in the $\beta_2m^{-/-}$ lpr mice provides compelling evidence that the majority of $B220^+$ DN T cells are derived from peripheral $CD8^+$ T cells that have been positively selected in the thymus. This conclusion is strongly supported by a very recent report that MRL-lpr class II knockout mice that lack $CD4^+$ T cells accumulate DN T cells in numbers comparable to those of MRL-lpr mice. The persistence of a small proportion of $B220^+$ DN T cells in both the anti-CD8

mAb-treated lpr and gld mice and the $\beta_2m^{-/-}$lpr/lpr mice suggested that a proportion of $CD4^+B220^+$ T cells may further downregulate the expression of CD4 and acquire a phenotype indistinguishable from $CD8^+$ T cell-derived $B220^+$ DN T cells. In untreated lpr and gld mice, we propose that only a small proportion of $B220^+$ DN T cells would be derived from $CD4^+$ T cells.

The accumulation of $CD4^+$ and $CD4^+B220^+$ T cells in the absence of $CD8^+$ T cells suggests that $CD8^+$ T cells normally may be involved in the regulation of $CD4^+$ T cell activation and survival. Others have reported that autologous MLR (AMLR) responses by $CD4^+$ T cells are improved in the absence of $CD8^+$ T cells and this may explain the strong AMLR response observed with cultured $\beta_2m^{-/-}$ lpr LN cells. The strong AMLR reactivity also implies that a high proportion of $CD4^+$ T cells in the $\beta_2m^{-/-}$ lpr LN are self-reactive. Continuous stimulation of these cells *in vivo* may contribute to the accumulation of activated $CD4^+$ T cells and $CD4^+B220^+$ T cells. The $CD4^+$ T cell subsets may accumulate not only because they are continuously activated but also because FasR/FasL-mediated deletion pathways are blocked. A manuscript describing these studies is in preparation.

III. Evidence for abnormal deletion of primed lpr and gld $CD4^+$ and $CD8^+$ T cells after exposure to super Ag in vivo

Superantigens combine with class II MHC to form ligands thata bind to particular Vβ elements of the TCR and induce strong proliferative responses that are followed by clonal deletion or clonal anergy. Included in the super Ag family are enterotoxins produced by Staphylococcus aureus. One of these, S. aureus enterotoxin B (SEB), specifically stimulates $V\beta7^+$ and$V\beta8^+$ $CD4^+$ and $CD8^+$ T cells. Recently, we used SEB as a stimulus for evaluating the immune responsiveness of lpr and gld T cell subsets *in vivo* and also for determining if lpr and gld T cells exhibit abnormalities in clonal deletion or anergy induction. Both young lpr and gld mice with minimal lymphadenopathy and older mice with well established lymphoproliferative disease were injected with SEB.

These studies showed that $V\beta7^+$ and $V\beta8^+B220^+$ DN T cells are unable to respond specifically to SEB *in vivo*. Instead, there is a weak non-Vβ restricted proliferative response followed by deletion. $CD4^+$ and $CD8^+$ $V\beta7^+$ and $V\beta8^+$ T cells proliferated strongly in response to SEB, and there was no evidence for abnormalities in tolerance induction. In contrast, some abnormalities were observed in deletion. Deletion of SEB-responsive $V\beta8^+$ and $V\beta7^+CD4^+$ T cells was normal in 4-6 wk old lpr and gld mice and impaired in older mice. Significant impairment in the deletion of $CD8^+V\beta7^+$ and $V\beta8^+$ T cells was observed in both young and old mice. Several conclusions can be drawn from these studies. First, the weak and non-specific proliferative response of $B220^+$ DN T cells implies that the accumulation of these cells may result predominantly from continuous production from $CD8^+$ precursors combined with abnormal deletion and/or survival rather than Ag driven proliferation. Second, FasR/FasL-mediated deletion mechanisms are not used exclusively for deletion of Ag-activated T cells in the periphery but may be required for efficient deletion of Ag-stimulated naive and memory $CD8^+$ T cells and memory $CD4^+$ T cells. Third,

FasR/FasL expression is not required for tolerance induction in $CD4^+$ or $CD8^+$ T cells. A manuscript describing this work is in preparation.

IV. Effects of *in vivo* treatment of lpr and gld mice with mAb specific for IFN-γ, TNF-α and TCR Vβ8

The progressive accumulation of memory-like $CD4^+$ and $CD8^+$ T cells in lpr and gld LN that we described previously. and the impaired deletion of these cells after exposure to bacterial superantigen suggests that FasR/FasL-mediated mechanisms may play an important role in regulating the number of memory T cells that persist after an immune response. We predict that an over-abundance of memory T cells with the potential to produce high concentrations of cytokines that promote inflammatory responses, B cell maturation and Ig isotype switching, may significantly enhance the probability of tissue injury and autoreactivity. Previously, we reported that both $CD4^+$ and $CD8^+$ T cells in mice with advanced lymphoproliferative disease produce high levels of IFN-γ and TNF-α after stimulation. To determine if these two cytokines influence the accumulation of $B220^+$ DN T cells or the development of autoimmune disease, we treated C3H-lpr and MRL-lpr mice chronically with mAb specific for IFN-γ and TNF-α. Neither treatment had an effect on the development of lymphoproliferative disease, but both treatments reduced the levels of circulating anti-ds DNA autoantibodies. Studies on the effect of Ab-therapy on kidney and skin disease in MRL-lpr mice are in progress.

Studies in our laboratory and several others have shown that there is a significant increase in Vβ8.3$^+$ T cells among $B220^+$ DN T cells and $CD4^+B220^+$ T cells. In other model systems of autoimmunity, including experimental allergic encephalomyelitis, there is evidence that V$\beta8^+$ T cells may play a prominent role in the development of autoimmunity. To further investigate the role of V$\beta8^+$ T cells in the development of lymphoproliferative disease and autoimmunity in lpr mice, we treated C3H-lpr and MRL-lpr mice chronically with anti-Vβ8 mAb. Although this treatment greatly reduced the proportions and numbers of V$\beta8^+$ T cells, it had no significant impact on the production or accumulation of $B220^+$ T cell subsets or the development of lymphoproliferative disease. Studies of the effects of depletion of V$\beta8^+$ T cells on autoantibody production and tissue injury are in progress.

Interactions between FasR and FasL clearly play a vital, but as yet poorly understood, role in preventing the accumulation of autoreactive lymphocytes and regulating the numbers of lymphocytes that survive after an immune response. In our future research we will continue to investigate the complex interactions among lymphocytes in lpr and gld mice that result in the selective accumulation of $CD8^+$ T cell-derived $B220^+$ DN T cells and the tight regulation of $CD4^+$ T cells and possibly B cells by $CD8^+$ T cells. The $\beta_2m^{-/-}$lpr and $\beta_2m^{-/-}$gld mice that we are presently inbreeding will be particularly useful for these studies. We have preliminary evidence that $CD4^+$ and $CD8^+$ dendritic cells may play an important role in supporting the production or survival of $B220^+$ T cell subsets. To further investigate this possibility, we will undertake a detailed study of dendritic cells in normal, lpr and gld mice. Dendritic cells will be isolated, characterized phenotypically and examined for their

stimulatory and veto functions. In collaborative studies with other investigators, we are breeding lpr and gld mice with defective expression of CD28, CTLA-4, B7-1 and B7-2. These knockout mice will be used for further investigating the block in signal transduction in $B220^+$ DN T cells and also for general studies of the importance of costimulatory signals in the selection, proliferation and survival of autoreactive T and B cells. Finally, studies are in progress to determine if lpr and gld are risk factors in the development of spontaneous or induced lymphoid tumors.

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Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1993 to September 30, 1994

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 identification and characterization of germ line and somatic mutations in the VHL gene. This gene appears to play a major role in the pathogenesis of sporadic clear cell carcinomas of the kidney.

We demonstrated that almost 60% of sporadic clear cell carcinomas of the kidney had somatic mutations in the VHL gene. The changes in the VHL gene were present in renal tumors, but not in corresponding normal tissues. With coworkers, we found that sporadic clear cell carcinomas of the kidney from patients in Germany and Japan also had somatic mutations in the VHL gene.

Missense, nonsense and frameshift mutations in the VHL gene were detected in sporadic clear cell carcinomas of the kidney; large deletions were not found. About half of the VHL mutations would be predicted to truncate the predicted protein product. Mutations in the VHL gene were found in both early and advanced stages of renal carcinoma.

Somatic mutations in the VHL gene appeared to be relatively specific for clear cell carcinomas of the kidney. Somatic mutations in the VHL gene were not detected in papillary renal cell carcinomas (n = 10), and in a variety of adult human solid tumors.

To define the mutation spectrum in the germ line of patients with VHL, we examined affected individuals from 114 families. We identified mutations in 85/114 (75%) of families. Missense, nonsense, frameshift and large deletion mutations in the VHL gene were detected. The mutations were distributed over a 400 nucleotide region in exons 1, 2 and 3.

VHL is a multisystem neoplastic disorder. The manifestations of VHL include retinal angiomas, central nervous system hemangioblastomas, bilateral renal cell carcinoma, pheochromocytomas and pancreatic cysts. Families vary in terms of which tumors they will develop. The most well-characterized difference between families is the predisposition to pheochromocytoma. In about 20% of the VHL families, pheochromocytomas may be present in gene carriers. Accordingly we divided VHL families into 2 types, VHL without pheo (type 1) and VHL with pheo (type 2).

The types of mutations found in VHL families with pheo differed from the types found in VHL without pheo. Virtually all (96%) of the mutations in VHL/pheo families were missense; in VHL families without pheos, different types of mutations were found.

Within the group of VHL/pheo families, several specific mutations were identified that were associated with distinct phenotypes. A 505 T to C mutation was associated with a predisposition to pheochromocytomas but not renal cell carcinomas or pancreatic cysts. A 712 C to T mutation was associated with a predisposition to both pheochromocytomas and renal cell carcinomas. The 712 C to T mutation and the

adjacent 713 G to A mutation appear to represent a mutation "hot spot"; these mutations were found in 10/26 U.S. and Canadian VHL/pheo families.

Coworkers in Germany have identified the 505 T to C mutation in 14/48 German VHL families. This prevalence of this mutation may represent a "founder effect".

We have identified additional families with papillary renal cell carcinomas. By contacting pathologists and urologists throughout the United Ststes we have identified one twin pair concordant for papillary renal cell carcinoma and about 20 individuals affected with mutiple papillary renal cell carcinoma without a family history of this illness.

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). A set of reagents for studying the role of NAP-1 and MCP-1 in animal models of disease has now been obtained. After cloning and expression of guinea pig NAP-1 and MCP-1, we now have polyclonal antibodies to these proteins. Relative to the possible role of endotoxin (LPS) in the development of acute respiratory distress syndrome (ARDS), we have begun studies on guinea pig NAP-1 and MCP-1 gene expression in response to intraperitoneal administration of LPS. Injection of 4 mg/kg of LPS resulted in histological changes consistent with LPS-induced lung damage. Northern blotting showed marked NAP-1 mRNA expression in lung but weak expression in heart, liver, kidney, and spleen. On the other hand, MCP-1 mRNA expression was strong in all the above organs except liver 1 hour after the injection. The same expression pattern was seen until 6 hours. We observed marked NAP-1 mRNA expression in human lung obtained from individuals who died after trauma; message was low or undetectable in other tissues. In contrast, marked MCP-1 mRNA expression was seen in heart, lung, skeletal muscle, kidney, and pancreas. Thus, the results obtained in guinea pigs after LPS injection were similar to that found in humans following trauma. A detailed study will now be done to correlate expression of the two cytokines at different times after LPS-injection with development of lung injury.

Antigenic stimulation of specifically sensitized leukocytes causes secretion of MCP-1 and NAP-1. To investigate the possible role of IL-2 (which is secreted by antigen-stimulated lymphocytes), we measured secretory responses of lymphocytes and monocytes, alone or in mixtures, to stimulation by IL-2. IL-2-induced secretion of MCP-1 by lymphocyte-monocyte mixtures greatly exceeded secretion by the individual cell populations. This led to studies on the capacity of medium from lymphocytes after 24 hrs of stimulation with IL-2 to cause monocytes to secrete MCP-1 and NAP-1. IL-2-lymphocyte conditioned medium was more effective than IL-2 in stimulating monocytes to secrete MCP-1 or NAP-1. The geometric mean concentration ratios for the two experimental conditions were 4-fold for MCP-1 and 14-fold for NAP-1. We then stimulated monocytes with combinations of lymphokines that were found in the IL-2-lymphocyte conditioned medium. We concluded that enhanced monocyte secretion of MCP-1 and NAP-1 could be accounted for by (1) synergistic action of IL-2 + TNF-α + IFN-γ; and (2) direct stimulation by GM-CSF. The low concentrations of MCP-1 and NAP-1 in the IL-2-lymphocyte conditioned medium relative to the high concentrations found after this medium is added to monocytes show that monocytes are the major producers of these chemoattractants via the IL-2-initiated pathway.

MSP is a member of a protein family, the members of which are secreted as precursors, which have no biological activity until the single protein chain is cleaved into α and β chains by specific serine proteases. Identification of pro-MSP convertases is therefore the key to understanding how the biological activity of MSP is controlled. We expressed ^{35}S-cysteine-labeled recombinant pro-MSP in MSP cDNA transfected Chinese hamster ovary cells and studied proteolytic processing of pro-MSP. Nanomolar concentrations of coagulation proteases kallikrein, factor XIIa or factor XIa cleaved pro-MSP to MSP within 30 min. Pro-MSP had no biological activity. After cleavage by kallikrein, biological activity was quantitatively comparable to that of natural MSP isolated from human plasma. These results support our hypothesis that MSP circulates as the biologically inactive precursor, and can be activated by enzymes of the intrinsic coagulation cascade. In view of the capacity of serum kallikrein to cleave and activate pro-MSP, we became interested in mouse nerve growth factor-γ (NGF-γ) and epidermal growth factor binding protein (EGF-BP), which are members of the glandular kallikrein subfamily of serine proteases. Each of these enzymes, at concentrations of 1 nM (2 orders of magnitude less than is required for cleavage by serum kallikrein) cleaved pro-MSP to MSP. Cleavage caused the appearance of biological activity, as measured by chemotactic activity of MSP for resident peritoneal macrophages, by MSP-induced macrophage shape change, and by stimulation of macrophage ingestion of C3bi-coated erythrocytes. These findings suggest the possibility of cooperative interactions between NGF-γ or EGF-BP and pro-MSP in inflammation and wound healing.

This was the first year of a 4-year CRADA with Toyobo of Japan to investigate the biological activities of MSP, with a view to possible diagnostic or therapeutic development. Toyobo has already produced purified recombinant pro-MSP in quantitities that are sufficient for in vitro experiments and for in vivo studies in small animals. Their MSP preparation is a mixture of pro-MSP, MSP and truncated α-chain. Since the biological activity of this preparation reflects the MSP content, it is probably suitable for exploratory in vivo studies.

We previously reported that serum from normal human subjects contains MCP-1 and NAP-1 immune complexes, comprising 1 IgG linked to MCP-1 or NAP-1. In contrast, free NAP-1 and MCP-1 are below the detection limits of our assays. In extending these studies on sera from 48 normal subjects, we found NAP-1 and MCP-1 immune complexes in all, with a highly significant correlation between NAP-1 and MCP-1 immune complex concentrations in individual sera. The mean complex concentrations were 330 $\pm$ 33 pM MCP-1-IgG and 220 $\pm$ 21 pM NAP-1-IgG. If we assume that the half-life of these complexes is comparable to the half-life of normal IgG (23 days), normal humans have a steady state production of NAP-1 and MCP-1 on the order of 30 picomoles per day. This is not a negligible amount if we relate it to the peak total circulating MCP-1 of 1000 pmoles that occurs in response to a strong inflammatory stimulus (intravenous LPS).

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

PROJECT NUMBER
Z01 CB 08575-21 LIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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:	PROFESSIONAL:	OTHER:
4.0	3.0	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). A set of reagents for studying the role of NAP-1 and MCP-1 in animal models of disease has now been obtained. After cloning and expression of guinea pig NAP-1 and MCP-1, we now have polyclonal antibodies to these proteins. Relative to the possible role of endotoxin (LPS) in the development of acute respiratory distress syndrome (ARDS), we have begun studies on guinea pig NAP-1 and MCP-1 gene expression in response to intraperitoneal administration of LPS. Injection of 4 mg/kg of LPS resulted in histological changes consistent with LPS-induced lung damage. Northern blotting showed marked NAP-1 mRNA expression in lung but weak expression in heart, liver, kidney, and spleen. On the other hand, MCP-1 mRNA expression was strong in all the above organs except liver 1 hour after the injection. The same expression pattern was seen until 6 hours. We observed marked NAP-1 mRNA expression in human lung obtained from individuals who died after trauma; message was low or undetectable in other tissues. In contrast, marked MCP-1 mRNA expression was seen in heart, lung, skeletal muscle, kidney, and pancreas. Thus, the results obtained in guinea pigs after LPS injection were similar to that found in humans following trauma. A detailed study will now be done to correlate expression of the two cytokines at different times after LPS-injection with development of lung injury.

Antigenic stimulation of specifically sensitized leukocytes causes secretion of MCP-1 and NAP-1. To investigate the possible role of IL-2 (which is secreted by antigen-stimulated lymphocytes), we measured secretory responses of lymphocytes and monocytes, alone or in mixtures, to stimulation by IL-2. IL-2-induced secretion of MCP-1 by lymphocyte-monocyte mixtures greatly exceeded secretion by the individual cell populations. This led to studies on the capacity of medium from lymphocytes after 24 hrs of stimulation with IL-2 to cause monocytes to secrete MCP-1 and NAP-1. IL-2-lymphocyte conditioned medium was more effective than IL-2 in stimulating monocytes to secrete MCP-1 or NAP-1. The geometric mean concentration ratios for the two experimental conditions were 4-fold for MCP-1 and 14-fold for NAP-1. We then stimulated monocytes with combinations of lymphokines that were found in the IL-2-lymphocyte conditioned medium. We concluded that enhanced monocyte secretion of MCP-1 and NAP-1 could be accounted for by (1) synergistic action of IL-2 + TNF-α + IFN-γ; and (2) direct stimulation by GM-CSF. The low concentrations of MCP-1 and NAP-1 in the IL-2-lymphocyte conditioned medium relative to the high concentrations found after this medium is added to monocytes show that monocytes are the major producers of these chemoattractants via the IL-2-initiated pathway.

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Publications

Gillitzer R, Wolff K, Tong D, M:uller C, Yoshimura T, Hartmann AA, Stingl G, Berger R: MCP-1 mRNA expression in basal keratinocytes of psoriatic lesions. J Invest Dermatol 1993;101:127-131.

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Rose CE, Juliano CA, Tracey DE, Yoshimura T, Fu SM: Role of interleukin-1 in endotoxin-induced lung injury in the rat. Am J Respir Cell Mol Biol 1994;10:214-221.

Skeel A, Leonard EJ: Action and target cell specificity of human macrophage stimulating protein (MSP). J Immunol 1994;152:4618-4623.

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

PROJECT NUMBER
Z01 CB 08577-09 LIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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
T. Kishida Visiting Fellow
I. Kuzmin Visiting Fellow

COOPERATING UNITS (if any)
Dr. Hiltrud Brauch, Tech Univ. Munich, Munich, Germany; Dr. Hartmut Neumann, Freiburg, Germany; Dr. Bryon Williams, Cleveland Clinic, Cleveland, OH; Dr. Tetsuo Noda, Cancer Inst., Tokyo, Japan; and Dr. Eamonn Maher, Cambridge Sch Med, England

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, MD 21702

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.0	5.0	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 project is to define genes involved in the pathogenesis of urologic malignancies. Our major accomplishments this year are 1) identification and characterization of germ line mutations in 85 von Hippel-Lindau disease (VHL) families, and 2) the detection of somatic mutations in the VHL mutations in the VHL gene in 57% of clear cell carcinomas of the kidney.

Major Findings:

We identified germ line mutations in 85/114 of VHL families. Clinical heterogeneity is a well-known feature of VHL. VHL families were classified into 2 types based on the presence of absence of pheochromocytomas. The types of mutations responsible for VHL without pheochromocytoma (VHL type 1) differed from those responsible for VHL with pheochromocytoma (VHL type 2). Fifty-six percent of the mutations responsible for VHL without pheochromocytoma (VHL type 2) were microdeletions /insertions, nonsense mutations, or deletions; ninety-six percent of the mutations responsible for VHL type 2 were missense mutations. Specific mutations in codon 238 accounted for 43% of the mutations responsible for VHL type 2. The mutations identified in these families should be useful in presymptomatic diagnosis.

In trying to establish correlations between specific mutations and phenotype it is helpful to examine large multigenerational families. Such families by providing a number of individuals with the same mutation, make it possible to accurately characterize the phenotypic effects of a particular VHL mutation. One large multigenerational family with a 712 C to T mutation had members affected with retinal angiomas, hemangioblastomas, pheochromocytomas and renal cell carcinomas. A second large multigenerational family (3127) with a 505 T to C mutation has frequent pheochromocytomas but no member affected with renal cell carcinoma.

Members of family 3127 came to the United States between 1725 and 1750 from the Karsruhe area of Germany and settled in Pennsylvania. Dr. Hiltrud Brauch and Dr. Hartmut Neumann have identified the 505 T to C mutation in 14 families from the Black Forest region of Germany; these 14 families have disease manifestation similar to those found in family 3127, pheochromocytomas but no renal cell carcinomas.

Localized and advanced tumors from 110 patients with sporadic renal cell carcinoma were analyzed for VHL mutations and loss of heterozygosity (LOH). VHL mutations were identified in 57% of clear cell renal carcinomas analyzed and LOH was observed in 98% of the samples. Moreover, VHL was mutated and lost in a renal tumor from a patient with familial renal carcinoma carrying the constitutional translocation, t(3;8)(p14;q24). The identification of VHL mutations in a majority of localized and advanced sporadic renal cell carcinomas, and in a second form of hereditary renal cell carcinomas indicates that the VHL gene plays a critical role in the origin of this malignancy.

We have identified additional families with multiple members affected with papillary renal cell carcinoma.

Publications:

Chen F, Kishida T, Yao M, Hustad T, Glavac D, Dean M, Gnarra JR, Orcutt ML, Duh FM, Glenn G, Green J, Hsia YE, Lamiell J, Li H, Wei MW, Schmidt L, Tory K, Kuzmin I, Stackhouse T, Latif F, Linehan WM, Lerman M, Zbar B. Germ line mutations in the von Hippel-Lindau tumor suppressor gene: correlations with phenotype. Hum Mutation, in press.

Crossey PA, Maher ER, Jones MH, Richards FM, Latif F, Phipps ME, Lush M, Foster K, Tory K, Green JS, Oostra B, Yates JRW, Linehan WM, Affara NA, Lerman M, Zbar B, Nakamura Y, Ferguson-Smith MA. Genetic Linkage between von Hippel-Lindau disease and three microsatellite polymorphisms refines the localization of the VHL locus. Hum Mol Gen 1993;2:279-282.

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Gnarra JR, Tory K, Weng Y, Schmidt L, Wei M, Li H, Latif F, Liu S, Chen F, Duh FM, Lubensky I, Duan D, Florence C, Pozzatti R, Walther MM, Bander NH, Grossman HB, Brauch H, Pomer S, Brooks JD, Issac WB, Lerman MI, Zbar B, Linehan WM. Mutation of the VHL tumor suppressor gene in renal carcinoma. Nature Genetics 1994;7:85-90.

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Richards FM, Maher ER, Latif F, Phipps M, Tory K, Crossey PA, Lush M, Oostra B, Gustavson KH, Green J, Turner G, Yates JRW, Linehan M, Affara NA, Lerman MI, Zbar B, Ferguson-Smith MA. Detailed genetic mapping of the von Hippel-Lindau disease tumor suppressor gene. J Med Gen 1993;30:104-107.

Richards FM, Crossey PA, Phipps M, Foster K, Latif F, Lerman MI, Zbar B, Affara NA, Ferguson-Smith MA, Maher ER. Detailed mapping of germline deletions of the von Hippel-Lindau tumor suppressor gene. Hum Molec Gen, in press.

Sekido Y, Bader S, Latif F, Gnarra JR, Gazdar AF, Linehan WM, Zbar B, Lerman MI, Minna JD. Molecular analysis of the von Hippel-Lindau disease tumor supressor gene in human lung cancer cell lines. Oncogene, in press.

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

PROJECT NUMBER
Z01 CB 08579-01 LIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cloning tumor suppressor genes (TSG) from human chromosome 3p

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,
CO-PI: M. Lerman and B. Zbar

Other:
F. Latif	Visiting Scientist
I. Kuzmin	Visiting Fellow
F-M. Duh	Research Associate, PRI
L. Geil	Research Associate, PRI
H. Li	Research Associate, PRI
M.H. Wei	Research Associate, PRI

COOPERATING UNITS (if any)
Dr. J.D. Minna, U. Texas, Dallas, TX; Dr. G. Klein & Dr. E. Zabarofsky, Karolinska Inst., Stockholm, Sweden; Dr. M. Dean, NCI-FCRDC, Frederick, MD; Dr. H. Smith, G. Brush Cancer Res. Inst., San Francisco, CA; Dr. P. Rabbitts, MRC, Cambridge, UK.

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, MD 21702

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8.0	4.0	4.0

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 ultimate goal of the project is to precisely map (within several hundreds or less kilobases) and clone TSGs located on 3p that are involved in the origin and development of a number of human malignancies, including carcinomas of the lung, breast, ovary, cervix, and kidney. By necessity and by design, this project is a collaborative effort and involves several laboratories in the U.S. and Europe.

Our major accomplishment this year is the identification of several nested homozygous deletions in a number of SCLC cell lines at 3p21.3 and one homozygous deletion in a breast carcinoma at 3p13. These deletions precisely define the physical locations of the respective TSGs and provide a rapid access and means to clone these genes.

The entire length (approximately 8Mb) of the homozygous deletion in the SCLC cell line U2020 previously described (R. Rabbitts, et al, Genes, Chrom. Cancer 2: 231-238, 1990 and F. Latif, et al, Genes, Chrom. Cancer 5:119-127, 1992) was cloned in overlapping YACs and the critical region narrowed to approximately 1Mb by using a set of ordered probes and the retention of heterozygosity approach.

Major Findings:

Previously, extensive cytogenetic and molecular deletion mapping has implicated defined region of chromosome 3p as harboring TSGs involved in the origin or development of several common cancers, notably carcinomas of the lung, breast, ovary, cervix, and kidney. More recently, the discovery of homozygous deletions on 3p21.3, 3p13, and 3p12 marked the physical location of the respective genes.

Our efforts are directed towards the molecular cloning of these TSGs associated with SCLC (3p21.3 and 3p12), and a subset of breast carcinomas (3p13).

Progress Report:

(a) The 3p21.3 TSG: two overlapping and three nested homozygous deletions have been identified and extensively characterized by construction of a physical map (PFGE) and YAC-P1-cosmid cloning. A 450 kb cosmid contig covering the critical region was established and transcribed sequences identified. To date 9 new genes were isolated, sequenced and their candidacy evaluated by mutation analysis (SSCP of exons followed by sequencing. Two known genes were positioned on the contig.

(b) The 3p13 TSG: RFLP deletion mapping combined with FISH have demonstrated that two separate regions on 3p are involved in subsets of breast cancers and a homozygous deletion at 3p13 was discovered. At this time a 1.5 Mb YAC clone was isolated and shown to represent the deleted region.

(c) The 3p12 TSG: The entire length (approximately 8 Mb) of the U2020 deletion (R. Rabbitts, et al, Genes, Chrom. Cancer 2: 231-238, 1990 and F. Latif, et al, Genes, Chrom. Cancer 5:119-127, 1992) is now cloned in overlapping YACs and a large number of ordered probes used to narrow the critical region to approximately 1 Mb.

Publications:

Chen LC, Matsumura K, Deng G, Kurisu W, Ljung BM, Lerman MI, Waldman FM, Smith HS. Deletion of two separate regions on chromosome 3p in breast cancers. Can Res 1994; 54:3021-3024.

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1993 through September 30, 1994

Research in the Laboratory of Mathematical Biology (LMMB) covers a broad range of theoretical and experimental studies of biological systems. These studies include molecular modeling, 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 diseases 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 using the supercomputing facilities at the Frederick Biomedical Supercomputing Center, FCRDC.

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 phyllogenetic comparisons. Searches for general trends involve comparisons of structures of related genes.

RNA secondary structures are predicted in correlation with sites involved in internal translational initiation in picornaviruses, infectious bronchitis virus, and pestivirus genomes. 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 are necessary for full appreciation of the richness of functions of RNA. A program is under development that makes plausible 3-D models suitable for graphical and numerical molecular modeling. Using this and manual computer graphics programs 3-D model of rev response element domain II was constructed, refined by molecular mechanical calculations and found consistent with experimental results (Le and Martinez).

Tools for efficient quantitative comparison and classification of 3-D protein data are based on principles from computer vision and robotics that objectively and automatically perform these functions. The technique is called geometric hashing. Early success at pairwise structure comparisons has now grown to permit rapid database comparisons. A concise representation of molecular surfaces is used with geometric hashing to perform docking between molecular surfaces. Flexible docking, using hinged motions, provides improved potential for drug discovery (Nussinov, Lin, Tsai, Wolfson and Fisher).

New analytical tools for studies of proteins and nucleic acids have been developed and implemented. Numerical methods aid in the prediction of secondary structures, splice sites, promoters, and recombination sites in nucleic acids. Graphical tools are developed to display, analyze and manipulate molecular structures. 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 through 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 information required for them to be located in the genome. Unlike several other prokaryotic recognition sites, the sequences at phage T7 promoters have twice the required information. Genetic experiments are being done to determine the source of this and other anomalies and to determine the structure of the promoters.

A graphical technique for showing sequence conservation is used to visualize the patterns at binding sites. The technique is superior to consensus sequences. These '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 using this and other information theory based 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 biomolecules are able to act precisely. For example, conventional models of the restriction enzymes have failed to explain why EcoRI is able to select only 5' GAATTC 3' from all other hexamer sequences. The theory explains this as a coding similar to the error correcting codes used in telecommunications. Further extension of the theory connects this result to the Second Law of Thermodynamics. Both results 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 have continued. These investigations are directed to probe into the interactions of complex carbohydrate structures of glycoconjugates with proteins and their influence on the cellular recognition processes.

Sugar donor/acceptor binding domains of glycosyltransferases are being analyzed by producing the protein in *E.coli* (Boeggeman, Balaji and Qasba). The recombinant proteins accumulate in inclusion bodies as insoluble proteins. A method has been developed to renature and regenerate the enzyme activity from the inclusion bodies which has been further modified to increase the efficiency of renaturation to approximately 90%. Analyses of the NH_2-terminal deleted and substituted proteins have shown that the first 129 residues of the 402 residue long β-1-4-galactosyltransferase are not involved in the enzymatic activity. NH_2-terminal deletions of different lengths, including the first 129 residues of the protein, do not affect the K_m's either for the sugar-nucleotide donor, UDP-galactose, or for the sugar acceptors, N-acetylglucosamine, chitobiose or chitotriose. However, deletion or substitution of Cys134 in the protein results in the loss of enzymatic activity without affecting its binding to UDP-galactose, N-acetylglucosamine or α-lactalbumin, suggesting that Cys134 is involved in disulfide bond formation in the protein that is essential for the enzymatic function.

Studies on the conformational analyses of complex carbohydrates of biological importance by molecular dynamics (MD) simulations are being continued with an aim to get a precise idea about all the accessible conformers and correlate the conformation with their biological activity (Balaji, Rao and Qasba). A conformation that has a low probability of occurrence cannot be observed by NMR and other available methods, and such conformations may be relevant for their binding to the protein or other macromolecules as is evident from the recent

results on the X-ray crystal structure analysis of protein-carbohydrate complexes. Our initial attempts to correlate the binding affinities of the oligosaccharide ligands to the asialoglycoprotein receptor (ASGP-R) with their preferred conformations derived by MD simulations were successful. These studies on the conformational analyses were extended to N-linked oligosaccharides that are involved in the biosynthesis of glycoproteins. The preferred conformations for the mannose oligosaccharide intermediates, generated during the biosynthetic processing of $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ structure, were deduced from the MD simulations. The existing biochemical data have been rationalized on the bases of the conformations of the these intermediates, thereby proposing the probable biosynthetic pathways for the processing of $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ structure. MD simulation of a heptasaccharide whose conformation in the crystal structure is known were carried out to test if the MD simulations access the conformation of the oligosaccharide that has been observed in the crystal structure of the lectin-carbohydrate complex, but was not predicted from NMR studies. These studies showed that the heptasaccharide conformation that is less frequently accessed during MD simulations, which was not proposed from NMR studies, is the one that is observed in the crystal. This less frequently accessed conformation of the heptasaccharide during MD simulation presents a better complementary surface to bind to the symmetry related lectin molecule in the crystal. Our MD simulations furthermore suggest that the orientation of the $\alpha 1,6$ arm is not only influenced by χ but equally by ψ, and if the heptasaccharide is not in this conformation while initiating the binding process, the conformation around Man_6-$\alpha 1,6$-Man_m will be altered by changing ψ to provide better complementary surface and to form additional hydrogen bonds with the protein at the expense of internal hydrogen bonds. Our results present a stereochemical explanation for the observed structures in the lectin-oligosaccharide complex crystals, and also reveal the range of conformations an oligosaccharide can access, the information which is vital for understanding carbohydrate-protein interactions.

Simulation, Analysis and Modelling of Physiological Systems. Development continued on the simulation, analysis, and modeling (SAAM/CONSAM) computer programs (Zech, Greif). We are also continuing to develop the SAAM/CONSAM software, we have modified the input and output portion of SAAM and CONSAM making the programs compatible with the Windows 3.1 GUI. Following this a rudimentary graphical user interface (GUI) consisting of single window with a command line was written and the CONSAM was converted to a Windows 3.1 application server; thus giving CONSAM the following properties: 1)can receive input from and send output to a windows GUI; 2)interact with other windows applications; 3)Compatible with Microsoft's basic family of windows; 4)Compatible with Microsoft Foundation Class 2.0 (MFC 2.0); and 5)compatible with Microsoft object language embedding (OLE 2.0).

In keeping with the renewed efforts of the federal government to make use of the "Information Super Highway" SAAM and CONSAM, have been made available, anonymously, over the INTERNET network (Zech, Greif) via the FTP (File Transfer Program) utility or over the public telephone network via Zmodem or Kermit using a 19,200 baud modem. The install procedures have been streamlined to make the instillation of SAAM and CONSAM much easer from electronic mail copies. We plan to participate in the "MOSAIC" networking cooperation.

A mutant form of HDL apoproteinAI, designated $apoAI_{Milano}$ has been examined using tracer methodologies (Zech). The kinetics and dynamics of both normal and mutant apoAI metabolism in subjects were performed. In normal control subjects radiotracer $apoAI_{Milano}$ was catabolized more rapidly than the normal radiotracer apoAI establishing that $apoAI_{Milano}$ is kinetically abnormal with an increased FCR. In contrast the normal ApoAI radiotracer had a normal FCR. In two heterozygous $apoAI_{Milano}$ subjects, both normal apoAI and $apoAI_{Milano}$ had an increased FCR compared to normal controls.

Apolipoprotein B metabolism in normal control subjects and patients with Familial Hypertriglyceridemia (FHT) and Familial Hypercholesterolemia (FH) has been examined using tracer methodologies and compartmental models (Zech). Results have been compared and contrasted to the paradigm for ApoB and LDL metabolism which has evolved from detailed examination of the low density lipoprotein (LDL) receptor. In humans, the liver is central to the synthesis and metabolism of apoB and we have asked questions about the relationship between changes in apoB metabolism and levels of hepatic cholesterol and cholesterol ester. Whereas normal subjects and HTG patients secrete apoB primarily as large, triglyceride-enriched, VLDL; heterozygous FH patients have an absolute decrease in apoB production and secrete nearly 40% of the apoB as smaller intermediate density lipoproteins (IDL). In normal subjects about half of secreted apoB is catabolized before conversion to LDL; and in HTG patients over 60% follow this route of catabolism. In these studies a significant proportion of the lipoproteins containing apoB are returned to the liver before passing through the LDL pool. In contrast, nearly all the apoB containing lipoproteins secreted are converted to LDL before uptake by the liver. These findings are explainable as an adaption to an increase in hepatic cholesterol ester content which must result from a change in hepatic cholesterol metabolism but not by the classic LDL receptor paradigm.

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, Jernigan). Also, this same general procedure of using regular lattice points to divide and define a conformational space has proven useful for investigating the binding of small peptides to larger proteins (Covell, Wallqvist and Jernigan) and should lead to new methods of drug design.

Molecular modeling has been proceeding in three areas: membrane proteins (Guy, Durell and Raghunathan), DNA helices (Jernigan, Zhurkin, Jiang and Raghunathan), and DNA-protein interactions (Zhurkin).

New structural models have been developed for the ion selective regions of voltage-gated sodium, calcium and potassium channels. Models have also been developed for all transmembrane segments of a series of channels that may have similar transmembrane folding motifs. These include several potassium channels (inward rectifying, ATP-activated, G protein-actived, and pH dependent), postsynaptic ATP-activated channels, amiloride-sensitive sodium channels, and mechanosensitive channels from E-coli. The work on antimicrobial peptides has continued; ways that mammalian cecropins interact with bacterial endotoxins have been modeled. A project to make the modeling process more quantitative and objective has been initiated in which bacteriorhodopsin and G protein-coupled receptors are being analyzed.

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 triple helix in

recombination is being investigated. Issues under study include symmetry and regularity, specific ion stabilization and interstrand interactions.

Image Processing

Parallel computation has continued to be an important part of our research effort. We have used our 16384 processor MasPar MP-2 to do research in RNA folding. This has involved the use of two algorithms, the genetic algorithm and the dynamic programming algorithm. The genetic algorithm produced better results than the dynamic programming algorithm in a structural study of the poliovirus (see below), in addition to pointing out an error in the dynamic programming code. The dynamic programming algorithm (with Jacob Maizel and Jih-Hsiang Chen) has been shown to be able to be run in a more cost effective manner on the MasPar than on the CRAY YMP folding sequences as large as 9200 nucleotides.

Massively parallel computation has also proven to be significant in the realm of sequence comparison. BLAZE has been running at the rate of 320,000,000 cell updates per second utilizing a version of the Smith-Waterman algorithm against the amino acid and nucleic acid databases. We have also been creating non-redundant databases on a weekly bases that are run with BLAZE. BLAZE may be reached via a new user friendly front-end program on the VAX. We have also begun experiments with a new sequence comparison program "MPsrch", which based on initial tests, appears to be running at speeds 2-6 times faster than BLAZE (with Jin Chu Wu, Mark Gunnell and Gary Smythers). We have seen as many as 1.3 Billion cell updates per second. This program also has more features than BLAZE.

We have begun an exploratory project with Cray Research Inc. involving the porting of code to their new massively parallel MIMD architecture, the T3D. This machine is purportedly capable of teraflop performance.

We have developed a new method for discovering motifs in protein sequences (with Dennis Shasha). The method finds active motifs composed of nonconsecutive segments separated by variable length "don't cares" without prior knowledge of their structures. The performance of the algorithm is quite good in terms of the number of correct PROSITE classifications.

Work has continued on the heterogeneous RNA structure analysis workbench (with Wojciech Kasprzak). The system permits a researcher to explore in detail various areas of interest concerning RNA secondary and tertiary structure. Improvements include further enhancements as well as the beginning of a port to Silicon Graphics workstations. Some of the enhancements include additions to the 2D histogram function which tighten the relationship with the molecular display functions. This has permitted examination of large databases of RNA structures. In addition, more functionality for tertiary interactions has been incorporated, and we have improved the robustness of the network communications in the heterogeneous environment. The entire system has been test-ported to a different site with a different network configuration and MasPar.

The workbench has been used in various collaborative studies:

The GA has been explored further by applying it to the 5' non-coding region or poliovirus RNA (with Kathleen Currey). We compared the results with the currently accepted model and found that the GA seems to be producing more accurate results than the dynamic programming algorithm. We also examined the integrity of the poliovirus structure by randomly introducing mutations throughout the 5' non-coding region. We found that highly persistent stems were all either functional/structural units themselves or in functional domains.

We have been examining a class of RNA's known as 10Sa RNA in regard to its activity specifically related to lambda bacteriophage (with David Friedman). We are exploring a phenomenon that apparently the 10Sa RNA acts either to directly or

indirectly facilitate release of C1 protein from its DNA target site of E. coli. This may explain why certain hybrid phages fail to grow under certain conditions.

We have been exploring in greater detail the dimerization site of HIV-1 RNA (with Ettore Appella and Eric Baldwin). We have concluded by various experiments which include gel shifts, ultracentrifugation, RNase mapping studies and computer prediction methods that the dimer site consists of a stem and loop structure where apparently nucleocapsid protein binds helping to form the dimer. Nucleocapsid protein NCp7 was found to bind to both a 44 nucleotide and a 19 nucleotide synthetic oligomer which adopt the proposed structure. In addition, structural motif comparisons were done across eight different strains of HIV-1, each showing the persistence of the motif in question.

The GELLAB-II software system is an exploratory data analysis system for the analysis of sets of 2D electrophoretic protein 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.

Collaboration has continued on the GELLAB-II technology transfer CRADA with CSPI Inc. (Billerica, MA) for a commercially available system GELLAB-II+. This will make this 2D gel exploratory data analysis technology more easily available to cancer researchers on inexpensive Microsoft Windows-NT PCs than on more expensive and complex UNIX workstations.

GELLAB-II applications this period include: Ongoing studies of Rett syndrome, serum dioxin study, and fetal alcohol syndrome (with J. Myrick, Centers for Disease Control/Atlanta); characterization of inducibility and role of metallothionein (MT) in intracellular changes of other proteins after MT induction and their role in cell proliferation and differentiation (with P. Hunziker, U. Zurich).

We are extending GELLAB-II analysis to handle differentially PCR expressed genes in Prader-Willi syndrome (PWS) (with P. Rogan, Penn State Med. School). The contrasting expression profiles of the cDNAS in patients with PWS relative to children with other growth hormone deficiencies may reveal potential effectors that were transcribed from imprinted genes and give a better understanding of these control mechanisms.

We have continued work on the Protein-Disease relational Database (PDD) system (with C. Merril, LGB/NIMH). The PDD includes literature-based data on proteins found in body fluids (serum, urine, CSF) which exhibit significant concentration changes with cancer and other disease states. Links of proteins in the PDD are made to 2D PAGE gels where feasible and to other genome databases accessible on the Internet. Some of these capabilities as well as concepts from our previous work on the IPS' "Xconf - image conferencing system" are being used to construct Internet accessible Mosaic-based client query interface to our PDD.

Membrane Structure and Function

The research goals in the Membrane Structure and Function Section (Robert Blumenthal, Dimiter S. Dimitrov, Anu Puri, Charles C. Pak, Hannah F. Elson, Min Zhai) are directed toward 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, Murine

Leukemia Virus, Vesicular Stomatitis Virus, Paramyxovirus, and 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 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 membranes and the wide opening which allows delivery of the mucleocapsid 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 maybe used as components of targeted systems negotiating entry of therapeutic agents 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. However, transmission of HIV-1 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 nine main projects, most of them in collaboration with scientists at NIH and its contractors. 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 (met, mos, bcl-2) 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 $GABA_A$ and glutamate receptors (Takagishi).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08300-21 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

SAAM, Development and Applications for Analogic Systems Realization

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

Loren A. Zech, M.D.	Senior Investigator Detail from OD, NHLBI	LMMB, NCI

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; Juergan Schafer, Klinikum Der Philipps-Universitat (Continued)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors
☐ (a2) Interviews

D

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 modified the input and output portion of SAAM and CONSAM making the program compatible with the Windows 3.1 GUI. Following this, a rudimentary graphical user interface (GUI) consisting of single window with a command line was written and the CONSAM was converted to a Windows 3.1 application server; thus, giving CONSAM the following properties; (1) can receive input from and send output to a Windows GUI; (2) interact with other window applications; (3) compatible with microsofts basic family of windows; (4) compatible with Microsoft Foundation Class 2.0 (MFC 2.0); and (5) compatible with microsoft object language embedding (OLE 2.0).

Further support for the conclusion that neutral lipid content of the liver is the major determinant of plasma apoB metabolism, is derived from our findings that when diets and drugs are administered, which decrease the hepatic concentration of hepatic cholesterol ester content, the FCR of LDL in FH patients increases to normal values because of an increased LDL receptor expression. These findings are explainable as an adaption to an increase in hepatic cholesterol ester content which must result from a change in hepatic cholesterol metabolism but not by the classic LDL receptor paradigm. How this pathophysiology of increased hepatic lipid content arises from the mutation of the LDL receptor gene is unknown. Further understanding of this tightly regulated metabolic system now shifts back to the investigation of cholesterol and cholesterol ester metabolism.

Cooperating Units (Continued):

Marburg, Marburg, Germany, Drs. H. Bryan Brewer and Daniel Rader of Molecular Diseases Branch, NIHLB; Richard E. Gregg, Squibb Inst. for Med. Res. Princeton, NJ; Dr. Blossom Patterson, Operations Research Branch; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; Dr. Kevin C. Lewis; Human Nutrition Laboratory, NCI; Dr. Andre J. Jackson, FDA, Rockville MD. Dr. Mary Mckenna, University of Maryland Medical School, Baltimore Maryland; and Drs. Barbra V. Howard & David C. Robbins of Medlantic Research Foundation Washington DC.

PROJECT DESCRIPTION

Project Description #1:

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 32-bit 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 DOS users and a thousand or more new users of the program. This technique also resulted in several disadvantages, such as more than 4MB of extended memory are required, and a math co-processor is required. This migration made extensive use of Virtual Control Program Interface Corp. (VCPI) methods, and VCPI programs are not compatible with Windows 3.1

The Windows 3.1 environment enhances DOS by allowing more than one application to appear to run simultaneously. Windows 3.1 does not support the 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 third of this migration was accomplished last year. A significant portion of the second third was accomplished in the present year.

The input and output functions of CONSAM have been further modified so that CONSAM can function as a DLL in the Windows 3.1 environment. This version was completely tested and found to yield identical answers to the DOS version of CONSAM.

Following this, a rudimentary graphical user interface (GUI), consisting of a single window with a command line, was written and the CONSAM DLL was converted to a Windows 3.1 application server. In other words, CONSAM has now become a full fledged Windows 3.1 application giving CONSAM the following properties: (1) can receive input from a windows GUI and send output to a windows GUI; (2) interact with other windows applications; (3) compatible with Microsoft's basic family of windows; (4) compatible with Microsoft Foundation Class 2.0 (MFC 2.0); and (5) compatible with Microsoft object language embedding (OLE 2.0).

In the future, we plan to continue the conversion of CONSAM to a complete Windows 3.1 program. This involves the construction of a set of completely new plotting tools which can function in a separate window. As the plotting routines now used

with the DOS version of CONSAM are written to the GKS standard, and there is no GKS server available for windows, the only choice is to rewrite this portion of CONSAM. We also need to build a graphical input device which will accept drawings of a compartmental model on the CRT and translate this into a description which is compatible with CONSAM. In short, while we have almost completed making CONSAM compatible with Windows 3.1, we have not yet taken advantage of the ability to use the windows 3.1 GUI.

In addition to having made several major adjustments and significant corrections to the input and output algorithms, we and the community of users have detected several dozen errors in the software, which have now been corrected. The above changes represent a beginning of the 32nd major revision of SAAM and the corresponding interactive user environment CONWIN.

(2) **SAAM Workshops & Distribution**: In the past year we have been involved in several workshops.

We participated in the Fifth Mathematical Models in Experimental Nutrition Conference: Vitamins, Proteins, and Methods, held at Purdue University, Fort Wayne, Indiana. We organized and conducted a compartmental modeling workshop for 75 participants starting the day before the meeting and running throughout this major meeting. Over 50 copies of the SAAM/CONSAM software were distributed to new users at this workshop.

We were invited to participate in a NATO Advanced Study Institute where we taught compartmental modeling for two weeks and distributed more than 50 copies of SAAM and CONSAM to new users from around the world.

In conjunction with the American Institute of Nutrition we organized a Sunday evening teaching session on the development of compartmental models for beginners preceding the 1995 FASEB Meeting. This will represent a one to one teaching experience, maximizing the results for the time relationship.

In conjunction with the American Physiologic Society, we participated in a computer demonstration area where several hundred researchers came to ask questions about SAAM and CONWIN, at the 1994 FASEB meeting, and plan to continue this participation at the 1995 FASEB meetings.

The improved sensitivity of mass spectrometers and the increased availability of labeled precursors with multiple mass shifts has increased the number of groups undertaking investigations using tracer kinetics. Because of the large number of requests for help with the kinetic analysis from European investigators, and in an effort to establish other centers in the collaborative effort, we organized a SAAM workshop in Marburg, Germany for European investigators. In 1993 this workshop was a big success, and we will participate in a much larger SAAM Workshop, sponsored by the German Government, to be held in Marburg, Germany, June 14-18, 1994. Over 40 German investigators are expected to attend and several hundred pages of notes have been prepared for this workshop.

Many copies of the SAAM/CONSAM software have been provided to the scientific community over the past 12 months. As pointed out in the past by the scientific coounselors, this effort is to help 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 databases, such as the lipoprotein, retinoid and selenium databases. As pointed out in the latest lab review, we should make an effort to streamline the distribution of software to other researchers in the field. We have extended the plan to continue distribution to the scientific community, both to individual scientist and to groups of scientist at modeling workshops. In keeping with the renewed efforts of the Federal government to make

use of the "Information Super Highway" SAAM, CONWIN, and the utility programs contained in this DOS disk set have been made available, anonymously (we do not record any information), over the INTERNET network via the FTP (File Transfer Program) utility or over the public telephone network via Zmodem or Kermit using a 19,200 baud modem. A complete copy or updates are available on disk from this source. The install procedures have been streamlined to make the installation of SAAM and CONSAM much easier.

Project Description #2:

Application of SAAM and CONSAM to the Simulation and Analysis of Bio-Kinetic Data. So that the Bio-kinetic data collected will be applicable to compartmental analysis, this effort includes chairmanship of the Radioactive Research Drug Committee where all tracer studies come under review for scientific merit. This effort also includes 800 hours of patient contact and primary responsibility, as the NIH authorized user, for 40 or more lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

Major Findings:

(1) In collaboration with Drs. Waldo Fisher & Peter Stacpoole, we continue to study Apolipoprotein B metabolism in normal control subjects and patients with Familial Hypertriglyceridemia (FHT) and Familial Hypercholesterolemia (FH). The biology of the low density lipoprotein (LDL) receptor has been examined in great detail, and a paradigm for LDL metabolism has evolved from the comparative studies of cholesterol metabolism in a variety of cells from normal subjects and patients with familial hypercholesterolemia (FH). These cells from FH patients lack a functional LDL receptor, exhibit diminished LDL fractional clearance, induction of the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, increased cholesterol synthesis, decreased cholesterol ester production, and depleted cholesterol stores. The observed decrease in the fractional catabolic rate (FCR) of LDL is attributed to the mutated LDL receptor gene product.

In the experimental animal model of FH (WHHL Rabbit) cholesterol ester stores are increased, while hepatic synthesis is decreased. In humans with patients with FH, HMG-CoA reductase is suppressed, and LDL-apoB production rate is increased. These findings are not consistent with the presently accepted paradigm as applied to *in vivo* hepatic cholesterol metabolism.

In humans, the liver is central to the synthesis and metabolism of apoB. We can ask questions about the relationship between changes in apoB metabolism, levels of hepatic cholesterol and cholesterol ester. We have examined this hypothesis using tracer kinetic methodology and compartmental models. Whereas, normal subjects and HTG patients secrete apoB primarily as large, triglyceride-enriched, VLDL; heterozygous FH patients have an absolute decrease in apoB production and secrete nearly 40% of the apoB as smaller intermediate density lipoproteins (IDL).

In normal subjects about 50% of secreted apoB is catabolized before conversion to LDL; in HTG patients over 60% follow this route of catabolism. In these studies, a significant proportion of the lipoproteins containing apoB are returned to the liver before passing through the LDL pool. In contrast, nearly all the apoB, containing lipoproteins secreted are converted to LDL before uptake by the liver. Although apoB secretion is decreased in FH subjects, total LDL production is increased compared to normal subjects or HTG patients. In other words, under basal conditions, the elevated LDL found in FH subjects is due to increased LDL production and decreased receptor-mediated catabolism.

Further support, for the conclusion that neutral lipid content of the liver is the major determinant of plasma apoB metabolism, is derived from our findings that when diets and drugs are administered (which decrease the hepatic concentration of

hepatic cholesterol ester content), the FCR of LDL in FH patients increases to normal values because of an increased LDL receptor expression.

These findings are explainable as an adaption to an increase in hepatic cholesterol ester content which must result from a change in hepatic cholesterol metabolism but not by the classic LDL receptor paradigm. How this pathophysiology of increased hepatic lipid content arises from the mutation of the LDL receptor gene is unknown. Further understanding of this tightly regulated, metabolic system now shifts back to the investigation of cholesterol and cholesterol ester metabolism.

(2) In collaboration with Drs. Roma, Gregg 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 of the dynamical lipoprotein, 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 ten 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 without apoAII lipoprotein) has been investigated by many laboratories. Investigation of the HDL fraction, containing apoAI, apoAII, LpAI:AII, has been neglected because there was no disorder in which this lipoprotein was changed. It is unclear which fraction of HDL participates in reverse cholesterol transport. While it is generally accepted that subjects with low levels of HDL with correspondingly low levels of LpAI are at increased risk of premature atherosclerosis, not all subjects with low HDL suffer from increased risk. Since the early studies of patients with Tangier disease, it has been postulated that the level of risk may be associated with the transport of apoAI through the system and not the resultant level of HDL. A kindred with low HDL and low risk of cardiovascular disease has recently been detected.

Molecular biology and protein chemistry studies have concluded that the apoAI from this kindred has a cysteine substituted for a arginine at location 173. This mutant apoAI has been designated $apoAI_{Milano}$. To determine the kinetics and dynamics of apoAI metabolism in subjects with this mutant apoprotein, radiotracer studies were performed. In normal control subjects, radiotracer $apoAI_{Milano}$ was catabolized more rapidly than the normal radiotracer apoAI, establishing that $apoAI_{Milano}$ is kinetically abnormal with an increased FCR. In contrast, the normal ApoAI radiotracer had a normal FCR. In two heterozygous $apoAI_{Milano}$ subjects, both normal apoAI and $apoAI_{Milano}$ had an increased FCR compared to normal controls. The estimated total production rate for apoAI in the $apoAI_{Milano}$ subjects was normal. This study further indicates that the rate of flow of apoAI through the plasma is somehow related to the level of reverse cholesterol transport carried out by the HDL, and that the level of reverse cholesterol transport can be high even when HDL levels are decreased.

(3) In collaboration with Dr. Kevin C. Lewis, Laboratory of Molecular and Nutritional Regulation, NCI-FCRDC: 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, their interaction, influence and ability to substitute for vitamin A functions. 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 suggests that the tissue distribution, metabolism and storage of retinol is completely rearranged by administration of this medication. A complete kinetic study is being designed to examine and compare tissue levels of retinol in control and 4-HPR treated animals with emphasis on both eyes and liver, 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 human subjects, retinol deficient patients such as abetalipoprotenemia and subjects with tumors.

(4) In collaboration with Dr. Blossom Patterson, Operations Research Branch, NCI; Drs. Phil Taylor & 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 is being applied to the main study. Subjects were on a controlled diet for three days prior to dosing, and twelve days thereafter. This allowed us to determine their total intake of selenium, and helped assure that they would be in steady state. A split unit design was chosen 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.

As the analysis is based on extending the pilot study model to include this data, 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. In other words, the possibility of tracer from the first study influencing the second study. Our first task was to detect and remove carryover effects, if any. A comparison of the plasma data for each subject showed that the tail of the plasma curve for the second study was higher than that for the first study. This was true regardless of fasting order. To account for this carryover effect, we used the model to simulate the amount of tracer remaining in the body after 90 days. We estimate that about 40% of the first dose remained at the time the second was given. Most of this was in the slowly-turning-over tissues. This has proved to be much more difficult than anticipated.

The model is being extended to account for the body burden of tracer selenium, as the oral dose of 200 micrograms of ^{74}Se sodium selenite dose tracer was large compared to the intake of tracer ^{74}Se, it was approximately the same size as the pool of ^{74}Se stored in the tissues. This has proved to be much more difficult than anticipated and has required further refinements in the proposed model.

A model for the analysis of the kinetics of 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 non-fasting 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 cannot 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 08303-22 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	MS&FS, LMMB, NCI

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Charles Pak, Ph.D.	IRTA	LMMB, NCI
Dimiter S. Dimitrov, Ph.D.	Visiting Scientist	LMMB, NCI
Hannah Elson, Ph.D.	Expert	LMMB, NCI
Min Zhai, M.D.	General Fellow	LMMB, NCI

COOPERATING UNITS *(if any)*

Dr. Hana Golding, CBER; Dr. Joel Lowy, AFFRI; Dr. Michel Ollivon and Marie-Therese Paternostre, CNRS, France; Dr. Lev Bergelson, Hebrew University, Israel; Dr. F. Booy, NIAMS; Yossef Raviv, NIDDK; Dr. S.J. Morris, UMKC, (Continued)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure and Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors
☐ (a2) Interviews

B

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

The research goals in the Membrane Structure and Function Section are directed toward 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, Murine Leukemia Virus, Vesicular Stomatitis Virus, Paramyxovirus, and 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 sort of molecular rearrangements occur after/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 membranes and the wide opening which allows delivery of the mucleocapsid 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):

Kansas City; Dr. D.P. Sarkar, Univ. of New Delhi, India; Dr. W. French Anderson, USC; Drs. J. Knutson, D. Remeta and A. Ginsburg, NHLBI.

PROJECT DESCRIPTION

Major Findings:

1. Conformational changes in influenza Hemagglutinin (HA).

Influenza virus hemagglutinin (HA) undergoes an acid-induced conformational change that mediates fusion of the viral and endosomal membranes. We found that the fusogenic state of HA adopts a *molten globule* conformation *in vitro* which is structurally and thermodynamically distinct from the native and denatured states. Specific characteristics of HA, that are consistent with those generally accepted as empirical evidence for a *molten globule* conformation, include retention of secondary structure, disruption of tertiary structure, reduced thermal unfolding enthalpy, increased hydrophobicity, and compact appearance. The conformational stability of HA from influenza strain X:31 was further 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^{0}C with an enthalpy change of ΔH_{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 $\Delta H_{cal}/\Delta H_{vH}$=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. However, the secondary structure of the low pH form of HA appeared to be stabilized with respect to thermal denaturation. The paradox of destabilized tertiary structure coupled with stabilized secondary structure is of great interest from a protein chemistry point of view. Moreover, these observations may have implications for the formulation of models proposed for the fusogenic conformation of HA.

2. Kinetics of the Low pH Induced Conformational Changes and Fusogenic Activity of Influenza Hemagglutinin.

We made the first rapid kinetics measurement of the low pH-induced conformational change of HA, by monitoring the decrease of the intrinsic tryptophan fluorescence intensity using stopped flow mixing techniques. The kinetics of the fluorescence decrease depended strongly on the pH. At a pH optimal for fusion, the decay was fast ($t_{1/2}$=120ms), and could be fitted to a monoexponential function. Under suboptimal conditions (higher pH) the $t_{1/2}$ was in the range of seconds to minutes. In addition, a slow component appeared and the fluorescence decrease followed a sum of two exponentials. The kinetics of conformational changes were compared to those of the fusion of influenza virus with red blood cell membranes as assessed by the R_{18}-dequenching assay. At optimal pH the HA conformational change was not rate limiting for the fusion process. However, at sub-optimal pH, the slow transition to the fusogenic conformation of HA resulted in slower kinetics and decreased extent of fusion.

3. Insertion of influenza HA into target membranes measured before and after fusion by photosensitized labeling.

We have investigated the association of HA with erythrocyte membranes by photosensitized labeling with [^{125}I]Iodonaphtylazide (INA). This technique relies

on the collisional energy transfer from a photosensitizing chromophore to INA, to selectively label proteins in the vicinity of the chromophore. Incubation of influenza virus with erythrocyte membranes containing chromophore and INA results in labeling of HA2 within 1 min following exposure at pH 5 and 37°C. We also examined photosensitized labeling of HA2 upon incubation of the X:31 strain of influenza virus with labeled erythrocyte membranes in a pre-fusion state (pH 5 and 4 C). Surprisingly, there was little HA2 labeling under those conditions, although incubation of the bromelain-cleaved fragment of HA (BHA), which contains the fusion peptide but not the transmembrane region, under the same conditions did result in rapid labeling of BHA2. We observed the rapid labeling of HA2 following exposure of intact virus-erythrocyte complexes at pH 5 and 37°C whether the INA was photosensitized by the non-exchangeable lipid dye DiO incorporated into the erythrocyte membrane, or by means of a fluorescent substrate (NBD-taurine) passing through the erythrocyte band 3 sialoglycoprotein which is the anion transporter. In the latter case, HA2 labeling decreased after an initial rapid rise. This observation suggests that the fusion site is close to the sialoglycoprotein and that INA photosensitized labeling may be used to assay protein movement following fusion.

4. Insertion of VSV G protein into target membranes measured before and after fusion by photosensitized labeling.

The VSV envelope glycoprotein (G protein) has an internal stretch of amino acids, which by mutational analysis has been designated as a putative fusion peptide. In order to characterize the putative fusion peptide, we have examined the interaction of VSV G with target membranes by photosensitized labeling with INA. With virus alone labeling was localized to the transmembrane domain. Digestion by various proteolytic enzymes yielded an INA-labeled ~7 kD fragment that was recognized by antibodies specific for the carboxy terminus of VSV G protein. We observed photolabeling under fusogenic conditions (pH 5.5, 37°C) as well as non-fusogenic conditions (pH 5.5, 4°C, or subthreshold pH levels). The non-fusogenic insertion was reversible as reneutralization of low pH-treated samples resulted in an absence of G protein labeling. We are currently examining what portion of the extracytoplasmic domain is labeled.

5. Reconstitution of VSV envelopes.

Viral envelopes, containing the G protein, were constructed by dissolution of the virus in the detergent, octylglucoside, followed by removal of the detergent. Using an assay based on the relief of self quenching of a lipid probe incorporated in the envelopes, we observed fusion with cultured cells with similar characteristics as intact virus.

6. The role of the cytoskeleton in influenza virus fusion.

We examined the influence of the physical state of spectrin, the major component of the membrane skeleton on fusion of influenza virus with red blood cells. Heat-denaturation of spectrin at 50°C results in an enhanced kinetics of fusion assessed by the fluorescence dequenching assay, whereas, the extent of fusion was not affected. Modifications of phospholipid asymmetry, membrane fluidity, as well as swelling behavior of red blood cells, were not responsible for the observed effect. We hypothesize that the cytoskeleton may affect membrane curvature necessary for fusion.

7. Kinetics of initial pore formation during cell fusion mediated by influenza HA.

We have measured the initial fusion event by sudden changes in fluorescence of a membrane potential-sensitive fluorophore using video rate multi-wavelength fluorescence microscopy. These changes were detected prior to either membrane or

cytoplasmic redistribution. Membrane lipid redistribution preceded redistribution of molecules of low molecular weight (M_r>340). This observation has important implications for the examination of hemi-fusion type models for HA-mediated fusion.

8. Fusion mediated by murine leukemia viral envelope constructs.

We are studying structure-function relationships in membrane fusion mediated by the Moloney murine leukemia virus (MuLV) envelope glycoprotein. We are making use of chimeric envelope glycoproteins constructed in Dr. French Andersons' laboratory. Of particular interest is a lipid-anchored construct which in the case of influenza HA promotes hemifusion, not complete fusion. Using fluorescent dequenching, we show that GPI-anchored MuLV envelope glycoprotein mediates hemifusion when incorporated into virions.

Description of AIDS Research

9. A trans-Dominant Mutation of HIV-1 env Blocks Cell Fusion When Present Either in the Same Cell as the Wild Type Protein or in the Opposing CD4-Containing Target Cell.

A recombinant vaccinia virus was constructed to express a mutant gp120-gp41, in which the second amino acid in the N-terminal region of gp41 is a polar glutamic acid instead of a hydrophobic valine. Recombinant vaccinia viruses expressing the mutant envelope protein are unable to cause fusion between infected cells and $CD4^+$ T lymphocytes. When both viruses infect $CD4^-$ cells simultaneously, there is a dose-dependent inhibition of fusion to $CD4^+$ cells with an increasing fraction of the mutant virus. Interestingly, when the opposing $CD4^+$ target cells are infected with the mutant virus, while $CD4^-$ cells are infected with wild-type virus, a similar inhibition of fusion is observed. This suggests that the mutated envelope protein does not need to reside in the same membrane as the wild type protein it inhibits. It may therefore be useful in preventing the initial infection of $CD4^+$ cells by HIV-1.

10. Kinetics of HIV-1 entry measured by escape from photodynamic inactivation of virus labeled with a membrane-bound dye.

Using the photodynamic inactivation technique, we have developed a new approach to measure the kinetics of initial stages of entry of HIV-1 into cells. At various times following the incubation of labeled HIV-1 IIIB with H9 cells, the mixture was illuminated and p24 was measured 3-5 days later. Upon illumination 0, 10 and 30 min, respectively, after incubation the viral output was still 20%, 30% and 70% of the un-illuminated control. The inability of photoinactivation to prevent infection after the relatively long incubation period indicates a very slow viral entry process.

11. A quantitative assay for HIV-1 envelope glycoprotein-mediated cell fusion.

We have labeled BJAB cells (TF228.1.16) stably expressing HIV-1 with a DNA stain (Hoechst 33342) and incubated them with PKH26-labeled CD4+ SupT1 or CEM cells. Following image capture using the blue and red filter sets, the images were overlaid and fusion was analyzed using Universal Imaging software. This method allows a precise determination of HIV-1 envelope glycoprotein-mediated fusion events.

12. Fusion of CD4 bearing plasma membrane vesicles (CD4-PMV) with HIV-1 envelope glycoprotein-expressing cells.

Incorporation of a fusogenic lipid (cardiolipin) into CD4-PMV enhanced their fusion activity (based upon lipid mixing) with HIV-1 envelope glycoprotein-expressing cells (TF288.1.16). A toxin (hygromycin) was incorporated into CD4-PMV by exposure of the vesicles to detergent in the presence of drug followed by detergent removal. The toxin-bearing CD4-PMV was able to specifically kill the TF288.1.16 cells as measured by thymidine uptake.

13. *Taq* vs. Pfu Polymerase for Identification of HIV-1 Envelope Glycoprotein Sequences with 3'- Mismatch PCR.

We found that the inability of *Taq* DNA polymerase to proof read DNA, as opposed to *Pfu* DNA polymerase, provides a way to identify known single base mutations in HIV-1 DNA encoding *env*. On the other hand, the ability of *Pfu* DNA polymerase to proof and correct DNA can be used to detect cloned HIV-1 *env* sequences in DNA without discriminating between wild type and single base mutations. Therefore, *Pfu* DNA polymerase should be used for screening for the presence of all HIV-1 sequences regardless of random mutations generated by HIV-1 reverse transcriptase.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08363-12 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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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

Molecular Structure

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors
☐ (a2) Interviews

B

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

The primary goals of this project are to develop structural and functional models of membrane proteins; with emphasis on ion channel proteins. In developing models of large integral membrane proteins, such as voltage-gated ion channels, we use a long term, iterative, hierarchical process. Our initial models are typically relatively imprecise and very hypothetical; e.g., our initial models of voltage-gated sodium channels predicted which segments span the membrane and which segments form functionally important components of the structure such as ion selective regions, voltage sensors, activation gates, inactivation gates, and drug binding sites. As these predictions are tested and as more data are obtained, we develop more precise models of specific regions of the proteins and extend the models to homologous proteins. Almost all our initial predictions about voltage-gated sodium channels have now been confirmed experimentally as have our extensions of these models to homologous voltage-gated potassium and calcium channels. Recently, we have developed new, more precise models of the ion selective regions and outer vestibules of sodium, calcium and potassium channels. These models are supported by numerous mutagenesis experiments and explain how tetrodotoxin and saxitoxin block sodium channels and how charybdotoxin blocks some potassium channels. It is difficult to develop structurally precise models of the entire transmembrane region of voltage-gated channels because they are so large; e.g., each of four potassium channel subunits has six transmembrane helices in addition to a hairpin structure that determines the channels selectivity. Recently we have begun to model ion channels that may have similar ion selective segments but that probably have only two transmembrane helices per subunit. These include inward-rectifying potassium channels, g-protein-activation potassium channels, pH-dependent potassium channels, ATP-activated channels, amiloride-sensitive sodium channels, and stretch-activated channels.

We have also continued our work on antimicrobial peptides and compounds in collaboration with Michael Zasloff's group and are examining how pig cecropin interacts with endotoxins.

PROJECT DESCRIPTION

Major Findings:

Perhaps our most important contribution to the field of membrane channels has been the postulate that the narrowest portions of some pores may be formed by segments that span only a portions of the membrane and that cannot be identified by conventional methods of predicting transmembrane topologies. These predictions have been confirmed for voltage-gated sodium, calcium, and potassium channels and are likely to be true for many other channels that are homologous to voltage-gated channels. In voltage-gated channels, short hairpin segments in the outer entrance determine the pore's selectivity and form binding sites for numerous drugs and toxins. Additional segments that traverse only a portion of the membrane appear to form activation and inactivation gates responsible for the opening and closing of these channels. In order to understand the molecular mechanisms responsible for channel gating, ion selectivity, and much channel pharmacology, it is crucial to understand the structures of these segments. Our recently modeling efforts suggest that the ion selective segments in sodium, calcium, and potassium channels have an alpha helical hairpin structure. The helices from the four subunits (potassium channels) or four homologous repeating segments (sodium and calcium channels) are tilted to form a large cone-shaped outer vestibule in which the ion selective region is formed by the residues connecting the helices. In sodium and calcium channels, the connecting segments are short and atoms of charged side chains interact with the ion as it passes through the pore. In potassium channels, the connecting segments are longer and backbone carbonyl oxygens interact with permeant ions to determine the pore's selectivity. These models are supported by mutageneous experiments from numerous laboratories and explain how saxitoxin and tetrodotoxin block sodium channels and how charybdotoxin and tetraethylammonium block potassium channels. A primary motivation for this work is to use the models to develop better drugs. Several pharmaceutical companies have initiated large programs to develop immunosuppressant drugs that block voltage-gated potassium channels crucial for mitosis in T cells. We have begun to use the structure of charybdotoxin and our models of how it interacts with potassium channels to design such channel blocking drugs.

To make the pore portions of ion channels structurally complete, it is important to include all to the transmembrane segments. This is difficult to do for voltage-gated channels because there are so many transmembrane segments; six per subunit in addition to the segments that determine the channel's ion selectivity. Inward-rectifying, ATP-activated, and G protein-activated potassium channels have a simpler motif with only two transmembrane helices in addition to the ion selective hairpin. We are developing models of the entire transmembrane regions of these proteins. In these models each subunit has a partial transmembrane hairpin on both sides of the membrane in addition to the two transmembrane helices. We are also using this motif to develop models of other types of channels including amiloride-sensitive sodium channels and their homologs, pH-dependent potassium channels, ATP-activated post-synaptic channels, and stretch-activated channels.

Publications:

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Guy HR, Durell SR. Structural models of the ion selective regions and outer vestibules of sodium, calcium, and potassium channels. In: Sokabe M, ed. Towards molecular biophysics of ion channels. Cambridge, U.K.: Elsevier, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08370-11 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Interactions in Globular Proteins and Protein Folding

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

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Oleg Ptitsyn, Ph.D.	Visiting Scientist	LMMB, NCI
Stewart Durell, Ph.D.	IRTA Fellow	LMMB, NCI

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Ivet Bahar, Bogazici University	Bernard R. Brooks, DCRT
Sanzo Miyazawa, Gunma University	Anders Wallqvist, PRI, FCRDC

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Molecular Structure

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.1	3.1	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors
☐ (a2) Interviews

B

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

A 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.

Subjects of other protein studies have included the molten globule, a folding intermediate, use of hydrophobicities to detect binding sites for peptides on protein surfaces, and development of a new matrix to use for sequence comparisons based on the empirical potential energies.

Simple new two- and three-dimensional models were studied. From these it was learned that the conformational entropy can readily cause the lowest energy form to be disfavored over some others. Sensitivity analysis was performed on specific interactions, and it was learned that it is remarkably easy to achieve large scale transitions between two extremely different protein folds.

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 a 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 occurring 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 more thoroughly the role of amino acids sequence on three-dimensional structure.

Generalizing the approach so that an unknown structure can be considered is 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:

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1994;66:467-81.
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Young L, Covell D, Jernigan RL. A role for surface hydrophobicity in protein-protein recognition. Prot Sci 1994;3:717-29.

Jernigan RL. A new approach to protein folding calculation. In: Doniach S, ed. Statistical mechanics, protein structure and protein substrate interactions. New York: Plenum Press, in press.

Jernigan RL, Young L, Covell DG, Miyazawa S. Applications of empirical amino acids potential functions. Molec Eng, in press.

Miyazawa S, Jernigan RL. Protein stability for single substitution mutants and the extent of local compactness in the denatured state. Prot Eng, in press.

Ptitsyn OB. Kinetic and equilibrium intermediates in protein folding. Prot Eng, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08371-11 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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Other Professional Personnel:		
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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
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LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Molecular Structure

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.2	4.2	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors
☐ (a2) Interviews

B

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

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 over 2 million conformations, 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. Simulations of X-ray fiber diagrams were carried out that gave excellent agreement with experiment. 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.

Quadruple strand helices and their interactions with monovalent cations were investigated. Relative preferences among these ions agreed with experimental reports.

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 was 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 using 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 (Raghunathan, 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 a 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, Raghunathan, Camerini-Otero, and Jernigan).

Quantum calculations of natural and modified base pairs and triples were carried out (Jiang, Raghunathan and Jernigan) to look for unusually strong and unusually weak ones that might have therapeutic use.

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:

Barber AM, Zhurkin VB, Adhya S. CRP binding sites. Evidence for two structural classes with 6-bp and 8-bp spacers. Gene 1993;130:1-8.

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Zhurkin VB, Raghunathan G, Ulyanov NB, Camerini-Otero RD, Jernigan RL. A parallel DNA triplex as a model for the intermediate in homologous recombination. J Mol Biol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08380-10 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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Lewis Lipkin, M.D.	Medical Officer	LMMB, NCI
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Chung-Jung Tsai, Ph.D.	Visiting Fellow	LMMB, NCI
Nickaloi Alexandrov, Ph.D.	Visiting Fellow	LMMB, NCI

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LAB/BRANCH

Laboratory of Mathematical Biology

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	5.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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 tolls are developed to display, analyze and manipulate molecular structures. 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):

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Kathleen Currey, M.D.	Guest Researcher	LMMB, NCI
Ruth Nussinov, Ph.D.	Guest Researcher	LMMB, NCI

Cooperating Units (Continued):

Drs. Daniel Fischer and Haim Wolfson, Tel Aviv Univ., Tel Aviv, Israel.

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 increases, 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.

Our continuing research on RNA structure prediction focuses on improving the power of predictive methods and applying them to problems in important biological systems. Structural motifs, that play a crucial role in the regulation of internal initiation of cap-independent translation in human enteroviruses, rhinoviruses, encephalomyocarditis virus (EMCV), Theiler's murine encephalitis virus (TMEV), foot-and-mouth disease virus (FMDV), hepatitis A virus (HAV), infectious bronchitis virus (IBV) and hepatitis C virus (HCV), were extensively sought in virion RNA sequences using our algorithms. Predicted large stem-loops with associated pseudoknot structures and sequence complementarity between virus RNA and cellular 18S RNA, correlated with functional sites for internal ribosomal entry in picornavirus, HCV and IBV viral RNAs (Le, et al.). Variations of these structures occurred in other viruses. This generalizes and refines the models of internal ribosomal entry previously obtained.

RNA 3-D structure prediction: We focused on using our methods for RNA folding, including thermodynamics-based and phyllogenetics-based ones for identification of functional elements in mRNA of human and animal viruses as the starting points for extending the methods to 3-D molecular models. These RNA models will be the basis for further studies on RNA catalytic and binding activities and to RNA-protein interactions.

An initial, detailed, 3-D model of domain II of the rev response element(RRE) of HIV is completed. The model was built using manual computer graphics tools, and refined by molecular mechanical calculations under several simulated conditions. Superior agreement with mutagenic and other experiments required use of explicit water and sodium ions in the calculation. This, of course, requires more computer time than implicit solvent approaches(Le, Pattabiraman). This model indicates bending of the RNA-helix, conformational distortion of the sugar-phosphate backbone at two predicted non-Watson-Crick base pairs(G:G and G:A), and widening of the major groove, all of which may be necessary for interaction with rev protein. These results agree with experimental results of others. The non-Watson-Crick base pairs are predicted to be stabilized by bridging water molecules and naked sodium ions between the bases and sugar rings. Recent NMR results confirm these predictions.

Major improvements are in progress and near completion on the RNA 2D 3D program by Hugo Martinez. These improvements make significant refinements of the conformations of base pairs and the ability to exchange data with other programs for molecular graphics, molecular mechanical refinement, and molecular dynamic simulations. By alternatively subjecting the model to refinement and large scale manipulation, a wide variety of conformations are examined and rational atomic coordinates are maintained. These improvements bring us closer to being able to model RNAs in sufficient 3D detail to engage in close correlations with experimental results.

Protein 3-D structure comparisons: We continue to apply computer vision and robotics based approaches to 3-D protein structure studies. These techniques are uniquely suitable to three-dimensional, structural problems. Specifically, we apply them to (i) comparisons of protein structures and searches of substructural motifs and to (ii) docking of complementary substructural surfaces.

(i) The residue-based 3-D structure comparison program and an associated graphic program that displays comparisons automatically, were further developed. The primary source of structural data, the Brookhaven Protein Data Bank(PDB) has many, nearly identical, structures and data ranging widely in reliability. We have used "residue-based superposition similarity" to cluster the data into two non-redundant representative data sets for use in further detailed study. Based on an iterative procedure consisting of five individual steps, 400 "structurally homologous" families and 265 "analogous" families were generated from 2798 protein chains(Fischer, Tsai, et al.).

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(Fischer et al.).

An important problem in understanding any finding of similarities between C-alpha coordinates of proteins or families, is to assess the significance of such matches. Real and "random noise" similarities are not resolvable on the basis of the size of a match and the r.m.s.d. of corresponding C-alpha atoms. For example, in the case of triose isomerase structures, called TIM barrels, a histogram of scores based on C-alpha coordinates does not resolve the TIM scores from the upper tail of the histogram of all scores. A new criterion, designated as the "environmental score", E, based on the surrounding environments of the C-alphas, was developed. A dissimilarity D(i) is calculated from the different atoms surrounding two corresponding C-alphas. The total dissimilarity, D, is the sum of D(i). The environmental score is calculated as: $E= C*L - D$, where C is a constant and L is the number of C-alphas in the matched region. A histogram of the E scores resolves the real TIM matches completely from the random background scores.

Work continues on methods to automatically detect motifs in the structures. One approach is based on pairwise comparisons of sequences. If structure A contains a structural motif that also occurs in B and C, the same subset of atoms of A will be involved in the similarities A-B and A-C. If the subset is large enough and occurs in many proteins, it is called a motif. A program is under development for detection of such motifs, and it is being optimized to achieve practical performance (Alexandrov et al.).

In order to relate sequence and structure data, it is useful to assign secondary structural roles to residues. A method is being explored in which this may be done automatically by comparing prototypical template alpha helices, beta sheets, and coil C-alpha coordinates with a protein structure. Assignment is based on the quality of the best superposition, using the geometric hashing and other

algorithms, between segments of a protein and the templates. We find that using three ideal linear helices and 15 six-residue beta-sheets as templates, a limit of 88% correct assignment is achieved.

(ii) The second project 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. For efficient use, an accurate and precise reduced representation of surface topology is required. This was developed and reported last year. In brief, it defines critical points on cap-, pit- and saddle-like surfaces that are used in the geometric hashing, docking procedure.

This face-center surface representation was demonstrated to be promising in the Proteins paper (Lin et al.). A more comprehensive survey confirms its potency for molecular recognition on co-crystal and solution NMR structures in unbonded complexes. Deviation from experimental results was frequently near 0.5 A r.m.s.d. for the best dockings.

In related work, preliminary progress was made to describe and classify protein surfaces in terms of a spectrum of radii of spheres that inscribe four surface caps or is tangent to four surface caps, simultaneously.

In examining the interfaces between protein subunits, it was noted that some of the classical backbone motifs, such a four helix bundles occurred across the interface. This work is being extended to search for a wider variety of such bridging motifs.

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 domains in proteins. Preliminary results using this approach have been very encouraging.

Protein and nucleic acid sequence comparisons and motif detection: Improvements were made in the programs, Gnsrch and Dasrch, to manipulate GenBank and protein sequence databases for retrieval of sequences and information based on annotative data included in the flat files. This is the only program available that does relational-type joins with the data in this form. A related program is being developed to find short sequence motifs in libraries. Output is in the form of an all-way comparison of counts that occur in one, two or a selected number, if the motif occurs in more than two sequences. It can create sub-libraries that always contain or lack motifs of a specific size or sequence pattern. It presently works only with nucleic acids but is being adapted to proteins.

Publications:

Bachar O, Fischer D, Nussinov R, Wolfson H. A computer vision based technique for 3-D sequence-independent structural comparison of proteins. Protein Eng 1993;6:279-89.

Fischer D, Norel R, Wolfson H, Nussinov R. Surface motifs by a computer vision technique: Searches, detection and implications for protein-ligand recognition. Proteins Struct Funct Genet 1993;16:278-92.

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Fischer D, Wolfson H, Lin S-L, Nussinov R. Three-dimensional, sequence-order independent structural comparison of a serine protease against the crystallographic database reveals active site similarities: potential implications to evolution and to protein folding. Protein Science 1994;3:769-78.

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Norel R, Fischer D, Wolfson H, Nussinov R. Molecular surface recognition by a computer-vision based technique. Protein Eng 1994;7:39-46.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08381-11 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:

Geoffrey Orr Scientific Applications Analyst PRI/FCRDC

COOPERATING UNITS *(if any)*

Dr. J. Myrick, CDC/Atlanta; Dr. C. Merril, LBG, NIMH; Dr. P. Rogan, Penn State Med. Sch.; Dr. P. Hunziker, Univ. Zurich; Drs. A. Partin and D. Coffey, Johns Hopkins

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:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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.)*

The GELLAB-II software system is an exploratory data analysis system for the analysis of sets of 2D electrophoretic protein 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. Collaboration is continuing on the GELLAB-II technology transfer CRADA with CSPI Inc. for a commercially available system GELLAB-II+ making this technology easily available to cancer researchers on inexpensive PCs.

GELLAB-II applications this period include: Ongoing studies of Rett syndrome, serum dioxin study, and fetal alcohol syndrome; characterization of inducibility and role of metallothionein (MT) in intracellular changes of other proteins after MT induction and their role in cell proliferation and differentiation. We are extending GELLAB-II analysis to handle differentially PCR expressed genes in Prader-Willi syndrome.

We have continued work on the Protein-Disease relational Database (PDD) system. The PDD includes literature-based data on proteins found in body fluids (serum, urine, CSF) which exhibit fold concentration changes with cancer and other disease states. Links of proteins in the PDD are made to 2D PAGE gels where feasible and to other federated genome databases accessible on the Internet.

PROJECT DESCRIPTION

Major Findings:

GELLAB-II project #1:

We are using GELLAB-II on a number of ongoing collaborative projects. Two-dimensional gel electrophoresis analytic methods are continuing to be developed and enhanced for the GELLAB-II system. A new sub-project is attempting to extend it to help identify differentially PCR expressed genes using the GELLAB-II composite gel exploratory database analysis software. Various minor enhancements and collaborator support was provided for the UNIX version of GELLAB-II.

Our technology transfer CRADA of the GELLAB-II system with CSPI Inc. of Billerica, MA has continued this year, but at reduced effort on our part. The major effort reported last year was used to jump-start the initial technology transfer phase. The CSPI CRADA will result in commercializing the current UNIX workstation research version of GELLAB-II with a new version called GELLAB-II+ running on less expensive Microsoft Windows-NT PCs. Such commercialization will result in wider use and better support of the GELLAB-II technology than we can provide.

Collaborations using GELLAB-II

Our primary GELLAB collaboration this year has again been with Dr. James Myrick of the Centers for Disease Control (CDC) in Atlanta. We have continued our collaboration investigating several large 2D gel databases focusing on a Rett syndrome study (77 gels in the original followed by 35 cases and 35 controls in triplicate more during this period); fetal alcohol syndrome (52 gels in the original study with more samples during this period). We are increasing the number of patient samples in the Rett and FAS as well as serum dioxin (20 in original study and 20 new samples) studies.

Much of the collaboration has taken place remotely over the Internet with data being analyzed with GELLAB-II at both sites. The Xconf remote image-conferencing software methodology we had previously developed has helped us in this collaboration.

The CDC 2D gel studies during this period include (1) to (3) below. A study (4) of induced changes with metallothionein (MT) expression, in our collaboration with Dr. Peter Hunziker's group at the Biochemistry Institute of the Univ. of Zurich, is of interest because of the role MT may play in understanding cell proliferation and differentiation. We are participating in a study (5) to identify prostatic secretions to aid screening and staging of men with prostate cancer with Dr. Alan Partin and Dr. Donald Coffey at Johns Hopkins.

(1) The 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 constructed a 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. A number of marker proteins were found using GELLAB, confirmed with additional statistical analysis of the database, and may be of diagnostic significance for Rett Syndrome. These findings were reported by several posters and in a paper by Myrick et al. The study is being repeated with additional samples from Johns Hopkins, with 35 patient and 35 control samples run as triplicate gels, before attempting identification of the marker proteins.

(2) The fetal alcohol syndrome (FAS) study, analyzing 12 blood sera from children with FAS and 8 control children, was analyzed with the GELLAB-II system and detected protein biomarker(s) of this syndrome. FAS affects 1:700 live births in

the U.S. It 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. Our early findings indicate several marker proteins (reported by Robinson et al.). We are in the process of repeating the study with more samples as well as identifying the markers found.

(3) A Serum Dioxin Study. This project involves persons with known body burdens of 2,3,7,8-TCDD (dioxin). Twenty previously analyzed human sera samples and 20 new samples are being analyzed by 2D, and quantified proteins are being 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.

(4) Induced changes with MT expression. This project involves using GELLAB-II by Dr. Peter Hunziker's group at the Biochemistry Institute of the Univ. of Zurich. MT, a transition metal-binding protein is inducible in vitro and in vivo by a variety of agents (transition metals [Zn, Cd, Cu], cytokines, alkylating agents, physical stress). It is suggested that the protein may play a role in detoxification mechanisms (in the case of metal overload) but its inducibility by the other, more physiological factors, also suggest an involvement of MT in trace element (Zn, Cu) metabolism, in cell proliferation and differentiation. Previously, a number of investigators (including their group) have been looking for basal and induced changes of MT expression and synthesis. Just a few attempts were made to obtain an overall view of intracellular changes of the occurrence and/or synthesis of other proteins after MT induction. Thus, the aim of this project is to have a closer look at differences in the appearance or disappearance of proteins other than MT. They have already started to analyze the protein patterns of two different human cell-lines (Chang liver cells, skin epithelial cells). We have isolated some spots from 2D-gels and have analyzed them by sequence analysis, proteolytic peptide mapping and mass-spectrometry. Proteins found with the gel analysis will then be analyzed and characterized by the methods described above. The results will then give information about the mechanisms that go on within the cells under basal and inducing conditions.

(5) Identification of prostatic proteins correlated with stages of prostate cancer may aid in the screening and staging of men with prostate cancer. We are starting to collaborate with Dr. Alan Partin and Dr. Donald Coffey at Johns Hopkins to help analyze their data using GELLAB.

Monitoring gene expression with differential. PCR gels using GELLAB project #2

We are continuing the collaboration with Peter Rogan (Penn State Med. Sch.) to develop new methods in GELLAB-II to help analyze 2D DNA gels using the differential display PCR technique. We have been developing extensions to GELLAB to help analyze differences in gene expression in lymphocytes from patients with Prader-Willi syndrome (PWS) who have been given somatotropin. PWS patients with growth hormone deficiency will be treated with growth hormone

and monitored. The differential display PCR technique separates and displays a profile of cDNA products synthesized from mRNA on long DNA sequencing gels. GELLAB will be used to identify consistent changes in specific cDNAs. The contrasting expression profiles of the cDNAS in patients with PWS relative to children with other growth hormone deficiencies may reveal potential effectors that were transcribed from imprinted genes and give a better understanding of these control mechanisms.

Protein-Disease Relational Database project #3

We are continuing the collaboration between the IPS and Dr. Carl Merril's group in the Laboratory of Biochemical Genetics, NIMH. The goal of this collaboration is to create relational databases for proteins in commonly sampled body fluids: plasma, spinal fluid and urine proteins during normal physiological and cancer or other disease states. These databases will be linked when possible to the high resolution 2D electrophoretic protein patterns that have been derived from each of these fluids as well as to genomic databases accessible over the Internet.

A number of individuals have initiated 2D gel databases concerned with proteins in body fluids. Anderson et al. pioneered in this effort with their serum and plasma databases, and Goldman and Merril with their cerebrospinal fluid database. Hochstrasser, is currently updating the plasma database part of the SWISS-2DPAGE and is correlating it with SWISS-PROT. 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 literature about plasma, serum, CSF and urine protein changes that have been determined as varying in physiological and disease states accumulated over the past two decades. While many of these protein changes were measured by techniques other than electrophoretic, that is by: enzymatic, immune assays, or in some cases merely interaction with specific substances and sedimentation rates, this data can be used as a basis for establishing a proteins' changes in diseases 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 effected 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. This type of query may provide relationships between diseases which may have otherwise seemed unrelated, 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.

Current PDD status:

The Protein-Disease relational Database (PDD) includes literature-based data on proteins found in body fluids which exhibit a N-fold protein concentration change with disease state. We see the initial use of the PDD by researchers who need to search for these types of changes. Later, it may become more useful clinically as part of a pre-screen where changes in several marker proteins could be used to search the PDD for possible associated disease conditions - or to check a suspected disease for protein markers associated with it. To demonstrate the feasibility of the concept in our prototype, we initially concentrated on the Acute Phase Proteins in serum. However, we are gathering data on other body fluid proteins - in particular concentrating on cancer and tumor body fluid markers.

The detection methods reported in the literature include various enzymatic and immune assays and do not usually depend on a protein's being detected, quantitated or identified in 2D gels. If its (pIe,MW) position in 2D gels is identified in genomic databases, we reflect that in the PDD database. We use fold change (abnormal value/normal value) to normalize protein concentration data.

We have changed the focus of the graphical user interface for the PDD from that of using X-windows on a stand-alone computer system (reported last year), to one using the World Wide Web (WWW) protocol and the National Center for Supercomputing Applications (NCSA) Mosaic graphical user interface (GUI). Mosaic currently runs on most Unix systems, Macintosh computers, and Microsoft Windows systems, over the Internet. Both the Mosaic GUI and the WWW server (which enabled us to set up our own server) are available free from NCSA and are supported by that group.

This new type of Internet-accessible GUI interface has several powerful advantages including: (1) wide access by large number of investigators using the Internet; (2) investigators do not need any special software (other than the Mosaic program which they probably would have anyway); (3) we don't have to maintain or deal with user problems associated with their GUI interface since it will be a standard NCSA Mosaic interface; (4) database data entry can take place in a distributed manner by any password-enabled collaborators whom we allow access.

Because the system is to be "open" to the world through the Internet, we had to add barriers in the PDD server software to protect it so that malicious users won't be able to destroy or corrupt the PDD database when they are accessing it when doing queries.

The relational database (RDBMS) we are using is being developed in-house because we are adding object-oriented extensions for image processing. The RDBMS is memory-based so it should run faster than a disk-based RDBMS (as most commercial RDBMS systems are). Geoffrey Orr has extended this relational database to support the PDD to a "threads" programming model so multiple users can make simultaneous requests of the database. This memory-based RDBMS architecture also lends itself to the multi-processor thread-based processing hardware which the next generation of computers are beginning to offer. This means that the RDBMS server should be able to scale to new hardware fairly easily. We have also extended the front-end SQL parser of the RDBMS to use yacc and lex in order to be able to handle more complex query expressions.

We developed form generation and processing software required for the WWW paradigm. Forms are used for data entry by the PDD maintainers as well as the general user performing database queries. In addition, we have integrated an interactive image facility to show protein spots in reference gels in response to queries or to specify proteins by clicking on spots.

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 Microsoft 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 to research laboratories where it will be commercially supported.

Publications:

Lemkin PF, Wu Y, Upton K. An efficient disk based data structure for rapid searching of 2D gel databases. Electrophoresis 1993;14:1341-50.

Myrick JE, Lemkin PF, Robinson MK, Upton KM. Comparison of the Bio Image VisageTM 2,000 and the GELLAB-II two-dimensional electrophoretic analysis systems. Applied and Theoretical Electrophoresis 1993;3:335-46.

Wu Y, Lemkin PF, Upton K. A fast spot segmentation algorithm for 2D electrophoresis analysis. Electrophoresis 1993;14:1341-56.

Lemkin PF. Representations of protein patterns from 2D gel electrophoresis databases. In: Pickover C, ed. The Visual Display of Biological Information. River Edge, New Jersey: World Scientific Publishers, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08382-11 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
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Kathleen Currey, M.D.	Guest Researcher	LMMB, NCI
Jacob V. Maizel, Jr., Ph.D.	Chief, Lab. of Math. Biol.	NCI
Wojciech Kasprzak	Senior Applications Analyst	PRI/FCRDC
Jin Chu Wu, Ph.D.	Senior Applications Analyst	PRI/FCRDC

COOPERATING UNITS *(if any)*

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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:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Massively parallel computation has continued to be a significant portion of the past years research effort. The 16,384 processor MP-2 with a 6 gigaflop peak performance capability has been used to further explore the algorithms that have been developed and adapted to the MasPar. These are the 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, we have continued to work on the heterogeneous RNA structure analysis system with further improvements in its graphical presentation capabilities, RNA database matching facilities, mutated structure generation and extensions to the MasPar interface, including the beginnings of a port of the system to a Silicon Graphics front end.

The above system in conjunction with gel shift experiments, RNase mapping studies and ultracentrifugation 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*.

A new algorithm for discovering motifs in protein sequences has been further developed and published. 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. The results, of running this algorithm on 698 groups of the PROSITE database, appear to be very encouraging.

PROJECT DESCRIPTION

Major Findings:

Massively Parallel Computation

During the past year massively parallel computation has continued to be a significant part of the current research effort. We have been utilizing, a massively parallel computer architecture for the Laboratory of Mathematical Biology and Frederick Biomedical Supercomputer Center. The MasPar MP-2 contains 16,384 processors and is capable of peak speeds of 6 gigaflops/second on single precision floating point and 70,000 MIPS on integer operations. 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 have been adapted to this architecture.

Continued experiments with two versions of the Genetic Algorithm (GA) for RNA folding on the MasPar are being pursued (4). 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. The GA discovered 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. In addition, we have applied the algorithm to the poliovirus (see below) which is a well studied virus with several published papers presenting its structure. The GA gives results that represent the accepted model more closely than the dynamic programming algorithm. We have also begun to explore the parameter space of the GA more closely to determine how the alteration of the input parameters affect the results.

As part of the massively parallel computational effort on the MasPar we have been utilizing BLAZE, a massively parallel version of the Smith-Waterman sequence comparison algorithm which is capable of performing amino acid comparisons at the rate of 320,000,000 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 are considerably slower. In addition, sequence alignments may be produced. In addition, the ability exists to vary PAM matrices and gap penalty parameters. We have established an automated updating procedure that creates non-redundant forms of the amino acid and nucleic acid databases. These databases are reloaded into the MasPar on a weekly basis. In addition, a user friendly front-end has been established on the Vax to allow queries to be easily submitted and batched to BLAZE (with Gary Smythers and Mark Gunnell).

We have recently installed on the MasPar, "MPsrch". This is a new sequence comparison algorithm that is expected to run about 2-6 times faster than BLAZE. In one test case we observed a comparison rate of 1.3 billion matrix cells per second, significantly higher than BLAZE. In addition, it is supposed to have improved sensitivity, much faster loading times and more flexibility in regard to features. For example, it is capable of multiple sequence queries, queries of DNA

against protein databases and vice-versa (with Jin Chu Wu, 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. We are exploring further enhancements to the algorithm (with Jih-Hsiang Chen and Jacob Maizel).

We are also continuing to explore 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 (with Jacob Maizel).

We have also begun to explore the architecture and performance characteristics of a new massively parallel machine, the CRAY T3D. This MIMD computer claims to have the potential for teraflop performance. We are currently working with Cray Research Incorporated in adapting some of our codes for this architecture. Initially, we will be utilizing the T3D emulator that runs on the CRAY YMP and then we will run the codes on an actual machine.

RNA Structure Analysis Workbench:

Work on the nucleic acid structure analysis system has continued in a variety of collaborations and directions. This system has been developed for the analysis of RNA secondary and tertiary structure and runs on a SUN workstation. Recently it has been successfully tested on an SGI front-end. 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 permits intelligent queries of relationships that exist in the RNA secondary and tertiary structure problem domain by utilizing various software/hardware complexes available at the Frederick Cancer Research and Development Center and elsewhere. Currently, the system 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, and recently an SGI utilizing one common mouse and window system that reduces the users need to know the various software/hardware complexes. Recent examples of additions to this heterogeneous capability include the integration of two forms of the genetic algorithm and the dynamic programming algorithm for RNA folding on the MasPar.

The user has the interactive capability to fold thousands of structures and to cluster these structures to determine which are similar and which are not, as well as which substructures are similar and which are not. 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 significant 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 at various local structural elements. The structure may be labeled in different ways so that the important area of current interest may be viewed.

Structures may be compared analytically, for example, using a Boltzman distribution as well as visually. The system continues to be enhanced to include more functionality (with Wojciech Kasprzak) thus allowing more functionality and broader access to the research community. The SUN and SGI versions of the system utilize 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.

Some recent additions to and tests of the system include:

(1) Successful tests of the system on Silicon Graphics platforms both R4000 and R4400 based systems;

(2) Successful porting of the complete system to a totally different site and network configuration, including a different MasPar for demonstration of the GA and visualization capabilities;

(3) Enhancements to the 2D stem histogram function incorporating the ability to handle multiple histograms for structural comparisons of different RNA sequences;

(4) Enhancements to the graphical interface between the 2D stem histogram and structural drawings to allow for frequency based color coded structure labeling;

(5) Storing and restoring of the dot matrices data structures for future retrieval and display of this data. This is particularly useful for matrices constructed from 1000's of structures;

(6) Incorporation of tertiary interaction functions to allow exploration of potential RNA tertiary interactions;

(7) Experiments with a CLIM base GUI interface, which will eventually replace some of the currently existing GUI;

(8) Improvement of the communications protocol to increase the robustness and error trapping in the heterogeneous environment.

Examples of System Use:

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.

Collaborative efforts (with Kathleen Currey) have continued in looking at the 5' non-coding region of poliovirus RNA. We applied the GA to predict the secondary structure of this region (submitted paper to NAR). When compared with the currently accepted model (Skinner et. al, 1989), the GA performs better than the dynamic programming algorithm (DPA), MFOLD (Zuker et. al.). Of the 21 stems represented in the model, the GA finds all 12 of the stems predicted by the DPA plus six in addition, so that the GA finds 18 of 21 stems. Utilizing the 2D STEM HISTOGRAM function at the 60% level in the computer workbench, the DPA predicts 20 stems not found in the model while the GA only predicts six stems not found in the model. A case could be made for three of those extra stems for each algorithm. The GA predicts the structural elements found in the currently accepted model of the 5' non-coding region of poliovirus at a high rate and predicts the elements for which there is biological/biochemical data support. Furthermore, using only single sequence structural analysis, it is able to derive correct structures and actually performs better than the DPA when utilizing its suboptimal structure feature.

We also examined the integrity of the poliovirus structures by randomly introducing mutations throughout the 5' non-coding region. It was noted that certain stems/structures persisted despite mutation (non-compensating) within that stem/structure, leading us to postulate a persistence factor. When applied to the DPA and the GA, the highly persistent stems are all either functional structural units themselves or in functional domains. When the threshold is lowered, the resulting correlation with the known model structure worsens with the DPA, however, the GA results remain more comparable with the model structure. Some of the questionable stems generated by the GA decrease in frequency. Whereas, the persistence procedure is able to select biologically significant structures from both the DPA and the GA, its better performance with the GA reflects the GA's better performance at predicting secondary structure. Perhaps regions of the genome, where structure is important, have evolved a certain robustness to withstand isolated mutations. It is known that point mutations are produced at a relatively high rate in poliovirus as a result of replicative errors, thus, it would be advantageous to have a certain resilience in functional/structural elements.

We are now looking at the 5' non-coding region of coxsackie virus in collaboration with S. Tracy (University of Nebraska Medical Center) and evaluating the structure in relation to a single base change of Coxsackievirus 3. This has been correlated with cardiogenicity in a mouse model. Preliminary results indicate that this switch may be related to RNA structure. Additionally, we plan to evaluate/optimize the Genetic Algorithm utilizing the 5' non-coding region of the remaining polioviruses and enteroviruses as a model and tool, hoping to gain further insight into the secondary structure of the 5' non-coding region.

We have begun to explore a class of RNAs known as 10Sa RNA in regard to its activity specifically related to lambda bacteriophage. We are examining an affect that apparently the 10Sa RNA acts either directly or indirectly to facilitate release of C1 protein from its DNA target site of *E. coli*. This may explain the failure of hybrid phages to grow under certain conditions. Inappropriate binding of the C1 protein may be the cause. We are currently utilizing the RNA analysis workbench to study the structure of several related RNA's, to try to determine whether the secondary structure of the 10Sa RNA's is responsible for this phage activity. Currently, we are seeing some common structural features in three forms of the RNA. These are being experimentally confirmed by suggested computer generated mutations (with David Friedman).

A new method for discovering motifs in protein sequences has been developed and has been tested (2, 3). The method involves an automatic two step process: (1) find candidate motifs in a small sample of sequences; (2) test whether these candidate motifs are approximately present in all sequences. The method finds active motifs composed of nonconsecutive segments, separated by variable length don't cares without prior knowledge of their structures, positions or occurrence frequency. The algorithm has been incorporated into a system called DISCOVER. This system then acts as a protein classifier. The classifier was applied to all 698 groups of related proteins documented in the PROSITE database. The performance of the algorithm was quite good in terms of the number of correct classifications. It may even suggest the existence of new families of proteins (with Dennis Shasha).

AIDS Related Research:

The genomic RNA is in the form of a dimer in all retroviral virions. Studies have shown that the retroviral genomes are composed of two identical RNA molecules joined non-covalently at sites near the 5' ends. In a number of retroviruses, including HIV-1, stretches of ~100 nucleotides known as the

dimer linkage structure (DLS) have been identified. Mutational studies have shown that the assembly of the DLS is important for the packaging of the viral specific RNA. Thus, it would appear that dimerization is linked to RNA encapsidation. In addition, during the viral maturation process, the polyprotein *gag* precursor is processed to small components, one of which is nucleocapsid (NC) subunit p7. It appears that the nucleocapsid protein greatly enhances the rate of RNA dimerization under conditions of lower ionic strength. In a recent paper (1) we have shown by extensive computer analysis and RNase mapping studies that the DLS region of HIV-1 RNA can be folded into a stem loop structure that is recognized by the NCp7 peptide 1-55. The stem and loop structures are highly conserved among independent HIV-1 isolates.

Recently, we expanded on this work by looking at a larger portion of the HIV-1 terminal region 1-498. This work has further confirmed our secondary structure model for the encapsidation process. NCp7 binds to both 44 nucleotide and 19 nucleotide synthetic oligomers which adopt the proposed structure as further demonstrated by utilization of the RNA structure analysis workbench and ultracentrifugation. Nucleotides critical for NCp7 binding, have been mapped by chemical interference to a single site located within the first loop nucleotides. We have also begun to explore the structure of the reverse transcription binding site as well as mechanisms that might be able to disrupt these structures in cell culture.

The determination of the RNA structure discussed above, used several features within the RNA structure analysis system. Most significantly, the folding programs, sequence mutators, structure visualization tools, taxonomy drawings, 2D stem histograms and structural motif matching functions. It is a further proof of the concept that this system can be used successfully with collaborative experimentation. In addition to the methodologies reported last time, we used the system to examine the top 5000 suboptimal structures of HIVHX2B and HIVMAL. A two-dimensional stem histogram function permitted the determination of the prevalence of individual stems in these suboptimal structures. Also, we used the system motif searching capabilities to search a database of structures to determine the prevalence of the proposed structure in eight different variants, HIVHAN, HIVHX2BR, HIVLAI, HIVNL43, HIVNY5, HIVJRCSF, HIVRF, HIVSF2. This study of approximately 323 nucleotides in each of the eight variants centered around the psi site includes the generation of the top 50 suboptimal structures. Thus, 400 structures of the variants are compared and analyzed. The vast majority of the predicted structures (~82%) includes both stem loop 1 and 2, however some diversity of stem and loop structure is predicted. Specifically, the 400 structures are searched using the function "Pattern Match" in the RNA workbench that reported all sites that contained GGUG and GGAG in hairpin loops.

In addition, we repeated our previous folding to assess the effects of differing sequences and target size. We folded HIVHX2BR over the psi region using 498 nucleotides, which began with TAR at the 5' end and extended into the *gag* coding region. The top 5000 structures were subjected to stem frequency analysis. Stem and loop 1 was observed 84.7% of the time, while stem and loop 2 was observed 95.6% of the time. Similarly, we looked at the top 5000 structures of HIVMAL, 513 nucleotides and found stem and loop 1 84% of the time and stem and loop 2 93% of the time.

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 1993;90:5219-23.

Wang JTL, Marr TG, Shasha D, Shapiro B, Chirn GW. Association Computing Machinery. In: Snodgrass R, ed. SIGMOD 1994 Conference. New York, NY: ACM Publishing, in press.

Shapiro BA, Navetta J. A massively parallel genetic algorithm for RNA secondary structure prediction. Journal of Supercomputing, in press.

Wang JTL, Marr TG, Shasha D, Shapiro B, Chirn G-W. Discovering active motifs in sets of related proteins sequences and using them for classification. Nucleic Acids Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08387-07 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Cellular Nanoanatomy and Topochemistry

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

Pedro G. Pinto da Silva, Ph.D.	Chief, Membrane Biology Section	LMMB, NCI
Other Professional Personnel:		
Victor Romanov, Ph.D.	Visiting Fellow	LMMB, NCI
Christina Risco, Ph.D.	Postdoctoral Fellow	PRI, FCRDC
Shen Rulong, M.D.	Scientific Associate	ABL, FCRDC
Eliana Munoz, M.T.	Research Associate	PRI, FCRDC
Jose Carlos Mirones, M.D.	Guest Researcher	PRI, FCRDC
Yoshiko Takagishi, Ph.D.	Guest Researcher	PRI, FCRDC

COOPERATING UNITS *(if any)*

I. Tsarfaty, I. Givol, G. Nasioulas, G.F. VandeWoude, J. DuHadaway, R. Zhou, S. Rong, D.L. Ewert, 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:	PROFESSIONAL:	OTHER:
6.0	6.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have continued our efforts in Topobiology, i.e., the localization of specific molecules in cells. To this end, we use a combination of electron microscopic and cytochemical methods. Most ultrastructural localization involved the use of immunogold labeling, and recently, in situ hybridization. These methods are combined with a system of freeze-fracture and immmunocytochemical approaches developed in our laboratory in the recent past. Our work involves the collaboration with numerous investigators within and outside the NIH, and our immediate research contractors. Projects related with the localization and cellular biology of oncogenes show that overexpression of the mos oncogene can induce apoptosis; they lead also to the intracellular localization of met and bcl-2 oncogene products (RuLong). We have obtained biochemical confirmation for our previous ultrastructural localization and migration of the laminin receptor LR67 and have provided the first electron microscopical localization of the gastrin receptor in stomach cell (Romanov). Recent work centers on the participation of integrins in cell motility (Romanov). We have continued the work on the localization of GABA receptors and, in collaboration with scientists at the Fidia Center for the Neurosciences, are starting the localization of glutamate receptors (Takagishi). We have used immunogold labeling to follow the entrance and intracellular transport of the main proteins of Moloney murine leukemia virus and completed a study on the ultrastructure of Type II pneumocytes (Risco). Our laboratory has also continued longstanding collaboration with scientists at the Laboratory of Parasitic Diseases, in particular the studying of the dynamics of capping of infection-related surface molecules in Leishmania and the activity of magainin-1 against Bonamia ostrae (Pimenta).

Cooperating Units (Continued):

G. Puia, E. Slobodyansky, E. Costa, H. Caruncho, 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, T. Copeland, Lab. Molecular Virology & Carcinogenesis, ABL/FCRDC; K. Fukasawa, G. Nasioulas, D.L. Evert, ABL/FCRDC; H. Yamamura, Research Inst. of Environmental Medicine, Nagoya University, Nagoya, Japan; M.E. Sobel, Lab. of Pathology, NCI, NIH; V. Castronovo, Univ. of Liege, Belgium; N. Tarasova, C.J. Michejda, ABL/FCRDC; S. Aznavuorian, M. Stracke, Lab. of Pathology, NCI, NIH; M. Goligorskiy, New York State Univ. at Stony Brook, Dept. Nephrology; A. Morva, E. Bachere, DRIM, INSERM, Montpellier, France; D.F. Smit, Dept. Biochem. Imperial College, London, England; S-K. Kwon-Chung, M. Parta, Clin. Mycol. Sect., Lab. Clin. Invest., NIAID, NIH; K. Fukasawa, ABL/FCRDC; D.L. Ewert, The Wistar Inst. of Anatomy and Biology, Philadelphia, PA; T. Fujimura, Lab. Biochem. Pharm., NIDDK.

PROJECT DESCRIPTION

Major Findings:

A. Overexpression of mos oncogene product in fibroblast cells induces apoptotic cell death preferentially during S-phase (S. Rulong, K. Fukasawa, J. Resau, P. Pinto da Silva, G.F. Vande Woude).

The mos protooncogene encodes a serine/threonine kinase and its expression is absolutely required for oocyte meiotic maturation (Sagata et al.). The study of the mos product in oocytes and transformed 3T3 cells revealed the association with tubulin, the spindle and spindle poles (Zhou et al., 1991) which led us to propose that Mos contributes to the formation of the meiotic spindle and spindle pole. Consistent with this hypothesis, Mos was found to be a potent activator of a mitogen activated protein kinase MAPK (Posada et al., 1993). MAPK has also recently been implicated in the formation of the spindle and spindle pole during meiotic maturation. More recently, we have demonstrated that v-Mos overexpression in Swiss 3T3 cells alters normal mitotic spindle pole assembly and induces a meiotic-like spindle apparatus (Kenji et al., 1994). After acute infection of Swiss 3T3 cells with Mo-MuSV, cells expressing high levels of v-Mos round up and detach from the monolayer (floating cells). We showed that v-Mos expression induces M-phase specific phosphoprotein phosphorylation events at non-mitotic stages of the cell cycle as well as phenotypes reminiscent of those seen in meiosis. For example, the mitotic spindle apparatus at metaphase is asymmetrically positioned adjacent to the cell membrane in the floating cells. Moreover, the floating cells consist of two cell populations, viable, growth-arrested population, and dead or dying cells. The viable floating cells are growth arrested at the 2C or 4C-phase of the cell cycle, but not in S-phase. Here, we have further characterized the dead or dying floating cell population and find that these cells show typical characteristics for cells undergoing apoptosis. Our results indicate that only cells in S-phase are immediately disposed to apoptotic cell death when v-Mos is overexpressed. A wide variety of physical and chemical stimuli have been shown to induce apoptosis, including DNA damage, withdrawal or addition of specific growth factors, and inappropriate expression of genes that stimulate cell cycle progression. Since many of these stimuli alter highly ordered cell cycle progression, it has been proposed that apoptosis may be a response to inappropriate cell cycle perturbation. We observe inappropriate chromosome condensation and M-phase phenotypes in floating cells and this may induce apoptosis on cells in S-phase. Our results suggest the possibility that apoptosis may occur specifically during S-phase of the cell cycle. (Cell Growh & Diff. 5:419-429, 1994)

B. **Immunogold cytochemical localization of oncogenes and of oncogene-related proteins**

1. **Met oncogene** (S. Rulong, I. Tsarfaty, S. Rong, J.H. Resau, G.F. Vande Woude, P. Pinto da Silva). The met protooncogene product (Met) is a member of tyrosine kinase growth family and its ligand is hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF mediates liver regeneration in vivo, induces differentiation of Madin-Darby canine kidney (MDCK) epithelial cells into branching tubules, and promotes epithelial cell mobility and invasiveness in vitro. Two lines of evidence suggest that Met is involved in the formation and maintenance of epithelial lumenal structures: (i) Met is expressed in epithelial cells bordering lumenal structures in a variety of tissues, including cells that border the mammary duct, and (ii) treatment of certain carcinoma cell lines with human HGF/SF (HGF/SF^{HU}) induces the formation of lumenal structures in vitro.

NIH 3T3 cells produce murine HGF/SF^{mu} endogenously and become highly tumorigenic through an autocrine mechanism when they are engineered to overexpress murine Met. Likewise, NIH 3T3 cells coexpressing Met^{hu} and HGF/SF^{hu} (HMH cells) are highly tumorigenic. Explants of HMH tumors cultured on glass formed lumenal structures that were indistinguishable from lumens formed by HGF/SF^{hu} treated epithelial carcinoma cells in vitro. This result was unexpected because lumen formation has not been observed with cells of mesenchymal origin. Lumenal structures were not observed with the parental NIH 3T3 cells or with MT cells, a control cell line overexpressing the Met^{hu} receptor. To determine whether HMH tumors form lumens in vivo, we reexamined the paraffin embedded tumor tissues by electron microscopy. Toluidine blue stained semithin section revealed carcinoma-like areas. Thin section observation of this area under the electron microscope detected some desmosome like junctions, that is, the characterization of epithelial-derived carcinoma. The same tumor region was positive stained by Met^{hu} antibody. These data provide strong evidence that some HMH tumor cells had converted to a carcinoma morphology (Tsarfaty et al., Science 263:98-101, 1994).

2. **Bcl-2 expressed using a retroviral vector is localized primarily in the nuclear membrane** (S. Rulong, I. Givol, I. Tsarfaty, J. Resau, P. Pinto da Silva, G. Nasioulas, J. DuHadaway, S.H. Hughes, D.L. Ewert). A complementary DNA for human bcl-2 was cloned into the replication competent avian retrovirus vector RCASBP, and the resulting virus was used to express human Bcl-2 protein at high levels in chicken embryo fibroblasts. The expression of Bcl-2 did not transform or significantly alter the longevity of the chicken embryo fibroblasts in the presence of normal amounts of serum. However, the expression of Bcl-2 blocked c-Myc-induced apoptosis in these cells. Fractionation of the infected chicken embryo fibroblasts indicated that the protein was distributed equally between nuclear and high density cytoplasmic membranes.

Immunofluorescence analysis by confocal microscopy and immunoelectron microscopy showed that the Bcl-2 protein was primarily associated with the nuclear membrane and with the endoplasmic reticulum. Reduced amounts of the protein were associated with other membranes in the cytoplasm. These data show that, in this system, the Bcl-2 protein associates with the nuclear membrane and intracytoplasmic membranes but is not preferentially associated with mitochondria.

C. **Biochemical confirmation of the expression of 67 kD laminin receptor and its putative 37 kD precursor in response to laminin. Immunogold double-labeling study of the localization of 67LR and galactose binding lectins (L-14 and L-31) (Romanov, Sobel, Castronovo, Pinto da Silva).**

Quantitative Western blot analysis was performed to confirm our previous immunocytochemical observations concerning the expression of 67 laminin receptors and their putative precursors in response to activation of suspended A2058 cells by soluble laminin. We demonstrated that the enhancement of the expression of 67 LR and 37 LRP was associated to increased concentrations of laminin. This increase

was inhibited by cycloheximide. Our findings confirm previous data showing that binding of laminin to receptors at the cell surface induces the production and transport to the cell surface of an additional pool of laminin binding proteins.

Double-labeling immunogold electron microscopy was performed to demonstrate the distribution of the two galactose-binding lectins L14 and L31 and of laminin receptors in human melanoma cells. Although the functions of these lectins are not fully understood, there is evidence that they are involved in growth regulation, cell adhesion, and cell migration. In addition, they appear to play roles in neoplasia and the immune response. It has been hypothesized that these lectins may be involved in the formation of mature 67LR from its precursor. Our double-labeling experiments did not show a close relationship between lectins and laminin receptors.

D. Immunogold localization of the gastrin receptors in the rat stomach tissue (Romanov, Tarasova, Michejda, Pinto da Silva).

We carried out the immunogold localization of gastrin receptors in stomach tissue using anti-synthetic peptide antibodies against intra- and extracellular parts of the receptor molecule. Gastrin receptors were found within granules of one type of enterochromaffin cells, as well as in parietal cells. Our study is the first successful localization of the gastrin receptor by immunogold electron microscopy. Granule localization of gastrin receptors in the enterochromaffin cells might represent a storage place for use under special physiological conditions.

E. Participation of integrin molecules in cell motility (Romanov, Aznavuarian, Stracke, Goligorskiy, Pinto da Silva).

To study the participation of integrins in tumor cell invasion under chemotaxis, we performed the immunocytochemical analysis to assess the distribution of vitronectin receptors in invasive cells. Our preliminary results show that, even in the same cell cluster (formed in proximity of the artificial membrane pore), cells localized closer to the pore demonstrated higher expression of vitronectin receptors, as compared with cells at the periphery of the cluster. We are now studying the participation of integrins in wound healing using an experimental model (wounded cell monolayer). We have used immunogold electron microscopy to show that $\alpha 6$ integrin is expressed on the leading edge of the cell, near the wound.

F. The expression of glutamate receptors in cultured neurons: a freeze-fracture immunocytochemistry (Takagishi, Caruncho, Yamamura, Pinto da Silva).

The expression and distribution of several glutamate receptor subunits are being investigated in neurons developing in culture. Label-fracture and fracture flip techniques combined with immunocytochemistry using antibodies raised against peptides corresponding to the C-terminal portions of the glutamate receptor subunits GluR1, R2/3, and R4 are applied to detect a different subcellular localization of subunits in cultured neurons from hippocampus and cerebellum. A special attention is paid to an association of synaptic membrane differentiation.

G. Nuclear import of murine leukemia virus nucleocapsid protein and integrase during acute infection of NIH 3T3 cells (Risco, Menéndez-Arias, Copeland, Pinto da Silva, Oroszlan).

The entry and intracellular transport of Moloney-murine leukemia virions inside mouse NIH 3T3 cells was followed by electron microscopy. Four viral proteins (matrix [MA, p15], capsid [CA, p30], nucleocapsid [NC, p10], and integrase [IN]) were located by immunolabeling using gold probes. The virus enters the cells mainly by endocytosis, and viral cores devoid of their envelope reach the nuclear membrane. Soon after exposure to the virus, NC and IN were detected entering the nucleus, where they are both targeted to the nucleolus. However, labeling asso-

ciated to MA and CA was found only in the cytoplasm. NC contains potential nuclear localization signals and could enter the nucleus as part of a nucleoprotein complex, associated to viral nucleic acid and integrase. Our results also suggest that nuclear membrane breakdown may not be required for import of murine leukemia virus preintegration complexes into the nucleus of acutely infected cells (Cell, submitted for publication).

H. Type II pneumocytes: intracellular membranous systems, surface characteristics, and lamellar body secretion (Risco, Romero, Bosch, Pinto da Silva).

Type II pneumocytes, the producers of pulmonary surfactant, have been extensively studied during the last 20 years because of the importance of their metabolism in lung function and integrity. The ultrastructural studies of the 1970s and 1980s have shown that these cells present unique elements. In this work, we used thin-section, freeze-fracture, and fracture-flip electron microscopy techniques to obtain new information on the ultrastructural peculiarities of isolated rat type II pneumocytes, focusing our study on the intracellular membranous systems and their interrelationships and microanatomy of their plasma membrane during secretory process. In thin-sections of pneumocytes postfixed with osmium tetroxide and potassium ferricyanide, we observed that lamellar bodies (LBs) are usually connected to membranes of the endoplasmic reticulum, and seem to emerge and grow from them. Unusual connections between the endoplasmic reticulum and mitochondria were detected, as well as numerous bar-like structures (BLSs), most of them in the early stages of development and often generating from the nuclear membrane. Membranes of the smooth endoplasmic reticulum, that closely follow the outlines of mitochondria, also appear to be the origin of some BLSs. Possible transition forms, BLS--LB, were also detected, although they were rare. New images of the surface of the pneumocytes and its changes during LB secretion, showed a segregation and clearing of membrane particles at the areas of LB extrusion. We propose that LBs can originate directly from membranes of the endoplasmic reticulum or from BLSs. An indirect participation of mitochondria appears possible. The plasma membrane of pneumocytes displays structural changes associated with the secretion of LBs, as visualized by a redistribution of intramembrane and surface particles (Lab. Invest. 70:407-417, 1994).

I. Nanoanatomy and topobiology of protozoan parasites

1. *In vitro* activity of the antimicrobial peptide magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis* (Morva, Pimenta, Pinto da Silva, Bachere, Mialhe). Magainins are peptide antibiotics with broad antibacterial and antiparasitic activities, originally extracted from the skin of *Xenopus laevis*. We investigated the effects of magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis*. Viability of purified protozoa was assessed microscopically by the uptake of the vital dyes acridine orange and ethidium bromide. Following a 60 min exposure to magainin 1, *Bonamia* viability was reduced in a dose-dependent manner. Within the 500 µg/ml peptide concentration, the parasite viability was reduced by 94%. Electron microscopy showed membrane damage and release of cytoplasmic organelles in the injured *Bonamia*. The study of magainin 1 activity against *Ostrea edulis* hemocytes did not show any morphological change in the host cells, and the peptide did not impair the capabilities of hemocytes to produce chemiluminescence when stimulated to phagocyte zymosan particles. The possibility to genetically transform molluscs to generate disease resistant organisms is currently under investigation. Antimicrobial peptides such as magainins may provide effective gene sequences to be manipulated. (Diseases of Aquatic Organisms, in press).

2. Lipophosphoglycan capping on leishmania surface (Pimenta, Pinto da Silva, Smith, Sacks). The major glycoconjugate over the Leishmania parasites is the LPG (lipophosphoglycan) that forms a dense glycocalyx which covers the entire surface of the cell, including the flagellum. During the infective stage, this molecule undergoes structural modifications, which promote its survival in the vertebrate

host. More recently, the first protein marker form metacyclic (the infective form) of the parasite has been characterized. This protein, termed gene B protein, is located on the cell surface, yet it lacks any hydrophobic sequence for membrane attachment. Unlike other surface proteins, such as the surface protease, gp63, which are masked by the LPG coat, the gene B protein is accessible to antibody-colloidal gold particles. The co-capping data presented in this study demonstrates its association with LPG. The LPG capping does not distributed the intramembranous particles or gp63. In contrast, gene B protein is also located on the LPG-capping areas. This clearly demonstrates the association between this protein and LPG. It is possible that gene B protein participates in the developmental modifications of LPG molecules during the metacyclicogenesis (Experimental Parasitology, in press).

J. ***HYP1*, a hydrophobin gene from *A. fumigatus*, complements the *rodletless* phenotype in *A. nidulans*** (Parta, Chang, Rulong, Kwon-Chung, Pinto da Silva).

Aspergillus fumigatus produces conidia that are highly dispersable and resistant to degradation. We have sought to analyze these properties by studying the rodlets, or hydrophobins, which form the outer spore coat protein. Degenerate primers based on hydrophobins in other fungi were applied to genomic DNA from *A. fumigatus* in the polymerase chain reaction. A product of this reaction with moderate homology to an *A. nidulans* gene, as judged by Southern hybridization, was chosen for further study. Cloning and sequencing revealed a gene with two introns which encodes a protein of 159 amino acids. Structural characteristics consistent with other fungal hydrophobin genes, especially conserved cysteine residues, are present. Its expression is limited to the developmental stages in which maturing conidiophores are present. This *A. fumigatus gene, HYP1*, was used to transform a mutant strain of *A. nidulans* that lacks rodlets. Transformants with a single copy of *HYP1* expressed a rodlet layer on their conidia, as observed by freeze-fracture electron microscopy (Infection and Immunity, submitted for publication).

K. **Double stranded RNA virus in the human pathogenic fungas *Blastomyces dermatitidis*** (Kohno, Fujimura, Rulong, Kwon-Chung).

Double stranded RNA viruses were detected in a strain of *Blastomyces dermatitidis* isolated from a patient in Uganda. The viral particles were spherical (mostly 44-50 nm in diameter) and consisted of about 25% dsRNA (5 kb) and 75% protein (90 kDa). The virus contained transcriptional RNA polymerase activity and it synthesized single stranded (ss) RNA *in vitro* in a conservative manner. The newly synthesized ssRNA was a full-length strand and the rate of chain elongation was approximately 170 nucleotides per minute. The virus-containing strain showed no morphological difference from virus-free strains in mycelial phase. Although the association with the presence of the virus is unclear, the virus-infected strain converted to the yeast form at 37°C, but the yeast cells failed to multiply at that temperature (Journal of Virology, submitted for publication).

Publications:

Caruncho HJ, Pinto da Silva P. Alterations in the intermediate layer of goldfish meninges during adaptation to darkness. J Anat 1994;184:355-62.

Caruncho H, Pinto da Silva P, Anadon R. The morphology of teleost meningocytes as revealed by freeze-fracture. J Submicrosc Cytol Pathol 1993;25:397-406.

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 1993;603:234-42.

Givol I, Tsarfaty I, Resau J, Rulong S, Pinto da Silva P, Nasioulas G, DuHadaway J, Hughes SH, Ewert DL. Bcl-2 expressed using a retroviral vector is localized

primarily in the nuclear membrane and the endoplasmic reticulum of chicken embryo fibroblasts. Cell Growth & Differentiation 1994;5:419-29.

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 1994;70:407-17.

Tsarfaty I, Rong S, Resau JH, Rulong S, Pinto da Silva P, Vande Woude GF. The met proto-oncogene mesenchymal to epithelial cell conversion. Science 1994;263:98-101.

Morva A, Pimenta PF, Pinto da Silva P, Bachere E, Mialhe E. *In vitro* activity of the antimicrobial peptide magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis*. Dis Aquat Org, in press.

Pimenta P, Pinto da Silva P, Smith DF, Sachs DL. *Leishmania major:* Co-capping of the differentially expressed gene B protein and the lipophosphoglycan on the surface of metacyclic promastigotes. Exp Parasitol, in press.

Romanov V, Sobel ME, Pinto da Silva P, Menard S, Castronovo V. Cell localization and redistribution of the 67 kD and α6 ß1 integrin subunits in the response to laminin stimulation: an immunogold electron microscopy study. Cell Adhesion and Communication, in press.

Rulong S, Zhou R, Tsarfaty I, Hughes S, Vande Woude G, Pinto da Silva P. Immunogold labeling of oncogenic and tumor related proteins. Microscopy Research and Technique, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08396-06 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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Other Professional Personnel:		
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Stacy Bartram	SIP	LMMB, NCI
Maria Alavanja	SIP	LMMB, NCI
Jamie Fenimore	SIP	LMMB, NCI
Leslie Strathern	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

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INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.75	2.0	1.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Shannon's measure of information is useful for characterizing the DNA and RNA patterns that define genetic control systems. I have 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. In another case, 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. My 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. Progress was made at all levels.

Cooperating Units (Continued):

Kenneth E. Rudd, National Library of Medicine, NIH, Bethesda, 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, Dept. of Biochemistry, 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; Vishnu Jejjala, Univ. of Maryland, College Park, MD; Gaisela Storz, NIH, Bethesda, MD; Bill Benish, Harbor Beach, MI; Betty Kutter, Evergreen State College, Olympia, WA; Soren Brunak, Neil's Bohr Institute, Univ. of Copenhagen, Denmark and The Technical Univ. of Denmark; Martine Couturier, Universite Libre De Bruxelles, Belgium; Olga Podgornaya, St. Petersburg, Russia.

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. Many of the projects are also related by the use of experimental molecular biology to test hypotheses arising from sequence or information analysis. This approach proves to be broadly applicable to understanding many molecular systems.

Molecular Machines

Vishnu Jejjala volunteered to work in the lab last summer and was able to confirm many of the mathematical steps involved in the theory. A review of molecular machine theory for the journal Nanotechnology was recently published.

Individual Information

In the individual information technique, individual binding sites are assigned an information content, measured in bits. This method has been successfully 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 my 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 carefully collected 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 will be testing the prediction this summer by gel shift experiments to determine if purified FIS protein can bind to the predicted FIS site.

Two Fold Excess Information Content: T7 Promoter Project

The goal of this project is to determine by experimental methods the important components of bacteriophage T7 promoters, because the sequences at these promoters are more conserved than is necessary for the sites to be found. We have frozen 1800 random clones and will have 2250 completed by this summer. After characterizing them for T7 promoter function, we will sequence the clones. The sequences and characterizations will be used to train a neural network to simulate recognition by the T7 RNA polymerase. This combination of bench work and computer analysis has never been attempted before. Our goal is to understand the informational and 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. We are looking for the predicted second binding protein using PCR amplification and magnetic bead technology to isolate promoter binding proteins from cell extracts. If successful, this will confirm the information theory method for predicting binding proteins. Using our variant T7 promoters we were able to demonstrate by an in vivo assay that gene product 3.5 from bacteriophage T7 probably interacts with the T7 promoters. We are performing experiments to determine if 3.5 is the predicted second binding protein. The sequence logo technique is giving us clues about what to expect and look for in this project but bench work is essential for a full analysis of systems with excess information content.

Three Fold Excess Information Content: F incD Repeat Project

We discovered that the incD partition region of the F plasmid has three-fold excess information. Paul Hengen has obtained the incD region, recloned it into a set of plasmids designed for detecting the three predicted proteins and confirmed its sequence. He has shown that the cloned region acts as a partitioning locus in vivo. He has attached the DNA to magnetic beads and used these to isolate proteins from F-plasmid containing cell extracts. He has successfully identified three proteins which bind to the incD region and is now proceeding with purification. Once that is completed, we will be able to do 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.

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 on this system was completed and published in the Journal of Molecular Biology, and one of the figures was published on the cover.

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 publication of X-ray images of the protein-DNA co-crystal. 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 are submitting two papers describing these results.

***E. coli* Ribosome Binding Site 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 1982, 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 are investigating this new anomaly in detail. To explain why the older data set had a higher information content, we are also looking at all the binding sites of the bacteriophage T4, in collaboration with Betty Kutter.

Other Binding Sites

A project to determine how RNA editing works continues with 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. She has created a set of synthetic binding sites and asked for help analyzing them. Storz and her colleagues did a large amount of experimental work on this system in the last year, and confirmed the information theory prediction that the protein binds to 4 major grooves. A paper is in preparation.

Olga Podgornaya of the Institute of Cytology, Russian Academy of Sciences in St. Petersburg Russia has discovered two proteins which bind to human *Alu* retroposon-like short interspersed repeat sequences. We are collaborating on a project to align *Alu* sequences and determine their information content, to see if we can predict more binding proteins.

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 make our analyses very difficult. I have written a paper which proposes a specification for an advanced sequence database which should solve these problems, and which would save much wasted effort world-wide. My paper on this subject was accepted, pending revision, by Rich Roberts at Nucleic Acids Research. I presented these ideas at a genomics meeting in the National Library of Medicine and will present them again at a meeting later this summer.

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., 1994) 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. We have 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

This continuing project is aimed at developing an information theory based multiple alignment program. Vishnu Jejjala has taken over this project and has been invited to work in Denmark this summer in collaboration with Soren Brunak.

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:

Papp P, Chattoraj DK, Schneider TD. Information analysis of sequences that bind the replication initiator RepA. J Mol Biol 1993;233:219-30 and Cover of Vol.

Schneider TD. Protein patterns as shown by sequence logos. In: Keller PR, Keller MM eds. Visual Cues - Practical Data Visualization. Piscataway, NJ: IEEE Press, 1993;64.

Barrick D, Villanueba K, Childs J, Kalil R, Schneider TD, Lawrence CE, Gold L, Stormo GD. Quantitative analysis of ribosome binding sites in *E. Coli*.. Nucleic Acids Research 1994;22(7):1287-95.

Schneider TD. Sequence logos, machine/channel capacity, Maxwell's demon, and molecular computers: a review of the theory of molecular machines. Nanotechnology 1994;5(1):1-18.

Rogan PK, Salvo JJ, Stephens RM, Schneider TD. Visual display of sequence conservation as an aid to taxonomic classification using PCR amplification. The Visual Display of Biological Information, in press.

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Schneider TD. Genetic patterns as shown by sequence logos. In: Pickover C ed. Pattern Book. World Scientific, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08397-03 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Conformational Analysis of High Mannose Oligosaccharides by MD Simulations.

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
Ballurupalli 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:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have extended our studies on the conformational analysis of N-linked oligosaccharides by molecular dynamics (MD) simulations with the aim to get precise information about all the possible conformers these oligosaccharides can access and thereby correlate the conformational preferences to biological processes. The preferred conformations were derived by MD simulations for the Asn-linked mannose oligosaccharide intermediates, generated during the biosynthetic processing of Man9GlcNAc2 to Man5GlcNAc2 structure by α1,2-linkage specific mannosidases. The existing biochemical data was rationalized on the bases of the preferred conformation of these intermediates. The two α1,6- and two α1,3-linkages in each oligomannose take different conformations suggesting that the derived oligosaccharide conformations based on the conformational preferences of the constituent disaccharide fragments does not always yield correct results. The Man9GlcNAc2 structure, unlike other oligomannoses, appears to take more than one distinct conformation around the core α1,6-linkage. These various conformations may play an important role in determining the processing pathways. Using the data on the preferred conformations of these oligomannoses and the available experimental results, possible pathways for processing Man9GlcNAc2 to Man9GlcNAc2 by α1,2-linkage-specific mannosidases have been proposed, suggesting that the processing of the precursor oligosaccharide during Asn-linked complex and hybrid glycan biosynthesis proceeds in a well defined pathway involving more than one α1,2-linkage specific mannosidase. Knowledge of the conformation, of the processing intermediates obtained from the present study, can be used to design highly specific substrate analogs to inhibit a particular mannosidase, thereby blocking one processing pathway without interfering with the others.

PROJECT DESCRIPTION

Objective:

(1) Conformational analysis of Asn-linked high mannose oligosaccharide structures, which are generated during processing of the core oligosaccharide structure of a glycoprotein.

During the biosynthesis of Asn-linked glycoproteins, the $Glc_3Man_9GlcNAc_2$ precursor is co-translationally transferred from dolichol pyrophosphate donor to the side chain of Asn residue at the glycosylation site. Different types of N-linked glycan moieties are generated from this precursor oligosaccharide through the combined action of various glycosidases and glycosyltransferases, localized on the rough ER and in the Golgi complex. Although the assembly of these oligosaccharides on proteins occurs in closely related pathways in many organisms, the processing of oligosaccharides differs from species to species. These different pathways of oligosaccharide processing are controlled by specificities of the glycosyltransferases and glycosidases. Many of the diseases and malfunctions have in fact been attributed to incorrect processing and/or biosynthesis of the carbohydrate moieties of glycoproteins.

Since the spatial and steric considerations of the oligosaccharide substrates play an important role in their processing by enzymes, the knowledge of their three dimensional structure is essential. Molecular dynamics simulations based on semi-empirical force field calculations are very useful in studying their conformations. In the present report, different types of oligomannose structures that are known to occur in glycoproteins and/or during the metabolic pathways have been studied by molecular dynamics simulations for 1000 ps. These simulations have provided information about the preferred conformations of these oligomannoses and also predict their processing pathways.

Major Findings:

In the present studies, MD simulations of several high mannose oligosaccharides (Figures 1 and 2) were carried out for 1000 ps with multiple starting conformations. The accessible conformations of these oligosaccharides have been derived by analyzing the interglycosidic linkages ϕ, ψ, and χ of all the saccharide residues, which determine the conformation of the oligosaccharide molecule, instead of deriving the conformation of oligosaccharide by studying only the constituent di- and trisaccharide fragments. The conformation of the exocyclic-CH_2OH groups had no significant effect on the overall conformation or "shape" of the oligosaccharide. As described below, the conformational preferences and the flexibility associated with the two α1,3-($Man_3 \rightarrow Man_m$ and $Man_{36} \rightarrow Man_6$) and the two α1,6-($Man_6 \rightarrow Man_m$ and $Man_{66} \rightarrow Man_6$) linkages are very different. Our previous molecular dynamics simulations of some of the oligosaccharide ligands of asialoglycoprotein receptor (Project number Z01 CB 08397-02 LMMB), also showed that two similar linkages in an oligosaccharide can have different conformational preferences.

Conformation around β-1,4-linkages.

The number of mannose residues do not have any significant effect on the conformation of $GlcNAc_2$- $GlcNAc_1$, the chitobiose core. The conformation of all the oligomannoses around the core β-1, 4-linkages remains nearly the same throughout the 1000 ps simulation period and the interglycosidic torsion angles ϕ_{g1}, ψ_{g1} and ϕ_m, ψ_m mostly fluctuate around 55°, 0° with deviations of ±30° (Figure 1). Hydrogen bonds from $GlcNAc_1$-O3 to $GlcNAc_2$-O5 and $GlcNAc_2$-O3 to Man_m-O5 are possible in all the oligomannoses.

Conformation around the α1,3-linkages.

In all the oligomannoses, α1,3-linkages show more flexibility than α1,6-linkages. Of the two α1,3-linkages, Man_3-α1,3-Man_m (ϕ_3, ψ_3) is more flexible than Man_{36}-α1,3-Man_6 (ϕ_{36}, ψ_{36}) (Figure 1). Force field calculations have shown that the iso-energy map, as a function of the torsion angles ϕ and ψ, comprises a large and small region for the Man-α1,3-Man disaccharide. All the five minima in the large region are seen to be accessed by the high mannose oligosaccharides studied in the present molecular dynamics simulations. The decrease in the magnitude of fluctuation of ϕ_{36}, ψ_{36} compared to ϕ_3, ψ_3 may be due to the fact that in these oligomannoses, the α1,3 fragment in the α1,6 branch is placed close to the chitobiose core in most of the conformations.

Conformation around the α1,6-linkages in G2M9 verses other oligomannoses.

There are two α1,6-linkages in high mannose type of oligosaccharides - one in the core between Man_6 and Man_m (ϕ_6, ψ_6, χ_6) and the second between Man_{66} and Man_6 (ϕ_{66}, ψ_{66}, χ_{66}) (Figure 1). In general, the inner or the core α1,6-linkage is much more rigid than the outer α1,6-linkage. In all the oligomannoses except G2M9, ϕ_6, ψ_6, χ_6 prefer values around -60°, 180°, -60° irrespective of the initial values. In G2M6b, χ_6 appears to prefer the 180° conformation also, since in simulations started with initial χ_6 = 180°, transition to the -60° conformation takes place only after about 700 ps. Interestingly, when χ_6 is around 180°, ϕ_6 fluctuates from 60° to -60° and when χ_6 changes to around -60°, ϕ_6 is restricted to only -60° with ±15° fluctuations suggesting that when χ_6 is around -60°, the 60° conformation is not allowed for ϕ_6. No significant change in ψ6 is brought about by the transition of χ_6. These results show that the conformational preferences of ϕ_6, ψ_6, and χ_6 are interdependent.

Unlike other oligomannoses, χ_6 in G2M9 does not show any change from the initial value. It fluctuates around either -60° or 180° without any transitions during the whole of 1000 ps simulation period. However, in one of the simulations with initial ψ_6 = χ_6 = 150°, ψ_6 fluctuates during the first 500 ps around -70° and χ_6 around -40° and then ψ_6 changes to -120° and χ_6 changes to 60°. However, ϕ_6 changes only from around -30° to around -50°. In a disaccharide fragment, generally the 60° conformation for χ in α1,6-linkages is not favored due to the unfavorable syn-axial interactions between the O4 and O6 atoms. In view of this, it is interesting to see the transition of χ_6 from around -40° to 60° after about 500 ps. The variation in ϕ_6, ψ_6, χ_6 is generally within about ±15° from the average values in all these structures. Removal of any one of the terminal α1,2-Man from G2M9 (i.e., Man_{223} or Man_{236} or Man_{266}) favors χ_6= -60° conformation instead of the 180° conformation (i.e., in $Man_8GlcNAc_2$ isomers).

Conformational preferences of Man_{66}-α1,6-Man_6 (ϕ_{66}, ψ_{66}, χ_{66}) are very different from that of Man_6-α1,6-Man_m (ϕ_6, ψ_6, χ_6). Unlike χ_6, which has a strong preference for the -60° conformation (with the exception of G2M9), the conformation of χ_{66} depends on the number of mannose residues. With the exception of G2M8a, in all the oligomannoses where Man_{66} is the terminal residue without any substitutions (G2M5, G2M6a, G2M6b, G2M7a, and G2M7b [Figures 1 and 2]), χ_{66} shows equal preference for the two gauche conformations 60° and -60° and during the simulation period of 1000 ps, transitions between the two conformations are observed. When χ_{66} is in the 60° conformation, the unfavorable syn-axial interactions between the O4 and O6 atoms of Man_{66} appear to be offset by a hydrogen bonding interaction

between O4 and O6 atoms and by the hydrophobic interaction between the pyranose ring non-polar hydrogen atoms of Man_{66} and the trimannosidic core. In both the cases i.e., 60° and -60° conformations, the variation of χ_{66} is only about ±15° from the average value. Surprisingly, χ_{66} = 180° conformation is not seen for any of these five oligomannoses. However, in G2M8a which also has Man_{66} as a terminal residue, χ_{66} accesses all three staggered conformations- 60°, 180°, and -60°. ϕ_{66}, ψ_{66} are correlated with χ_{66}, when χ_{66} is around 60°, ϕ_{66} and ψ_{66} are around -60° and -150°. On the other hand, when χ_{66} is around -60°, ϕ_{66} and ψ_{66} are around 0° and 180°. Once again this shows that ϕ, ψ, and χ around α1, 6-linkages are interdependent. The variation in ψ_{66} is more when χ_{66} is -60° than when it is 60°. In G2M6c and G2M7c where Man_{266} is linked to Man_{66}, χ_{66} prefers only the 60° conformation. ϕ_{66} and ψ_{66} favor two sets of values around 30°, -90° and -60°, -150° in G2M7c and in G2M6c around -50°, 120°. G2M8b, χ_{66} takes both -60° and 60° conformations, whereas in G2M8c, only the -60° conformation is preferred. These results suggest that compared to the inner α1,6-linkage, the outer α1,6-linkage is more flexible and this may have implications in oligosaccharide processing in ER and Golgi complex.

Conformation around the α1,2-linkage.

α1,2-linkage occurs in all the oligomannoses studied here except G2M5. Although all the α1,2-linkages favor a conformation around ϕ, ψ = -40°, 0°, the degree of variation is different in different oligomannose structures. However, ϕ_{23} occasionally shows transitions to the 60° conformation in these structures. In general, ϕ_{223}, ψ_{223} and ϕ_{266}, ψ_{266} show similar conformational transitions as ϕ_{23}, ψ_{23}. Of the three terminal mannose residues, Man_{236} on the middle branch is held more rigidly than Man_{223} and Man_{266} in all the structures simulated here (ϕ_{236} = -55°±20°; ψ_{236} = -15°±15°) since it is placed close to the core except in one of the conformations of G2M9.

Effect of the amino acids around the glycosylation site on G2M9 processing.

The behavior of ϕ_6, ψ_6, χ_6 is very important since the conformation around the core α1,6-linkage mainly determines the "shapes" of these oligomannoses. As described earlier, unlike other oligomannoses which show an overwhelming preference for a conformation around 180°, -60° for ψ_6, χ_6 irrespective of the number and positions of mannose residues, G2M9 favors more than one distinct conformation around the core α1,6-linkage. These distinct conformations of G2M9 appear to be crucial in determining the oligosaccharide's overall conformation and may decide the pathway through which G2M9 is processed to G2M5 during glycoprotein biosynthesis. Amino acid residues around the glycosylation site may preferentially stabilize one of the possible conformations of G2M9.

Processing of G2M9 to G2M5.

After $Glc_3Man_9GlcNAc_2$ is transferred from dolichol pyrophosphate to the Asn residue of a nascent polypeptide chain, glucosidases I and II remove successively the α1,2- and α1,3-linked glucose residues forming G2M9. This is further processed by α1,2-linkage specific mannosidases to generate G2M5, the initial substrate for the biosynthesis of hybrid and complex type oligosaccharides. In the oligomannoses which contain both Man_{236} and Man_{266}, Man_{236} is closer to $GlcNAc_1$ than Man_{266}, except in some conformations of G2M9. Man_{223} is always placed away from the core. Thus, in all the oligomannoses, Man_{223} and Man_{266} are more accessible to mannosidases and are likely to be cleaved before Man_{236}, except in one of the conformation of G2M9. Hence, if Man_{236} is not cleaved from G2M9 in the first step,

it will be cleaved only after the other three α1,2-linked mannoses are removed. Based on the data obtained from the present MD simulations on the preferred conformations of the oligomannoses, three possible pathways for the processing of G2M9 to G2M5 can be envisaged as has been shown in figure 1 and figure 2.

When both ψ_6 and χ_6 in G2M9 prefer a conformation around -60°, Man_{266} is placed close to the chitobiose core and Man_{236} is away and exposed. Hence, Man_{236} can be removed first forming G2M8b. Since ψ_6 in G2M8b favors a totally different value, removal of Man_{236} from G2M9 brings about a conformational change in the oligosaccharide and makes Man_{223} and Man_{266} more accessible for mannosidase to cleave. ϕ_3 also changes from positive region (G2M9) to negative region (G2M8b). G2M5 can then be generated from G2M8b in two ways: (1) either Man_{266} is removed first followed by Man_{223} and Man_{23} (G2M8b → G2M7a → G2M6a → G2M5) or Man_{223} is removed first followed by Man_{266} and Man_{23} (G2M8b → G2M7c → G2M6a → G2M5). However, the possibility of Man_{23} being removed before Man_{266} cannot be ruled out, i.e., G2M7c → G2M6c.

G2M5 can also be generated from G2M9 by alternative pathways. When ψ_6 and χ_6 favor the 180° conformation in G2M9, only Man_{266} is easily accessible for mannosidases. In such a case, removal of Man_{266} from G2M9 will lead to the formation of G2M8a. Weng and Spiro (1993) have recently demonstrated the presence of an α1,2-linkage specific mannosidase in the ER of rat liver which specifically cleaves Man_{266} from G2M9. α1,2-linkage-specific mannosidase can successively cleave Man_{223} and Man_{23}, before Man_{236} (G2M8a → G2M7b → G2M6b → G2M5) as the latter is placed close to the core in both G2M7b and G2M6b. G2M9 can also assume a conformation with ψ_6 = 180° and χ_6 = -60° in which case the accessibility of Man_{266} is reduced. In such a case, Man_{223} will be removed first to generate G2M8c. Because of the reasons stated earlier G2M8c can also lead to the formation of G2M7b with the removal of Man_{266} and leaving Man_{236} still intact. Subsequent removal of this Man_{236} then follows the removal of Man_{23} (G2M7b → G2M6b → G2M5). Removal of Man_{23} before Man_{266} from G2M8c is also possible (G2M8c → G2M7d → G2M6b). Of the two α1,2-linked mannose residues in G2M7b (Man_{23} and Man_{236}), Man_{23} is cleaved before Man_{236} since Man_{236} is protected from mannosidases through its interaction with the core chitobiose. This is corroborated by the fact that the rate of cleavage of Man_{236} is much slower than that of Man_{223}, Man_{266} and Man_{23} (Schweden & Bause, 1989) and the rate of cleavage of Man_{236} increases on removal of the terminal GlcNAc residue of the core chitobiose (Bause *et al.*, 1992). Spiro and coworkers have characterized an endomannosidase which specifically hydrolyses the α1, 2-linkage between Man_{223} and Man_{23} when at least one of the three glucose residues is still linked to Man_{223} i.e., the conversion of $Glc_{[1-3]}Man_9GlcNAc_2$ to G2M8c (Lubas & Spiro, 1987). In fact, mutant cells lacking glucosidase II, the enzyme which hydrolyses the α1,3-linked glucose residues from $Glc_2Man_9GlcNAc_2$ intermediate, have been shown to utilize this endomannosidase to generate G2M8c (Moore & Spiro, 1992) and thus may process the oligosaccharide using this pathway. The processing of G2M9 to G2M5 by jack bean α-mannosidase has been studied *in vitro* by Tomiya *et al.*, (1991) and it proceeds via G2M8a, G2M7b, and G2M6b in agreement with the pathway proposed above. Figures 1 and 2 show that there are no common intermediates between the pathway which proceeds via G2M8a/G2M8c and the one which proceeds via G2M8b. This implies that the processing of G2M9 to G2M5 proceeds in a well defined sequence and not in a random manner. The MD simulations suggest that the particular conformation of G2M9, which is preferentially stabilized by the protein, will set the pathway by which G2M9 is processed to G2M5.

Occurrence of several mannosidases in ER and Golgi.

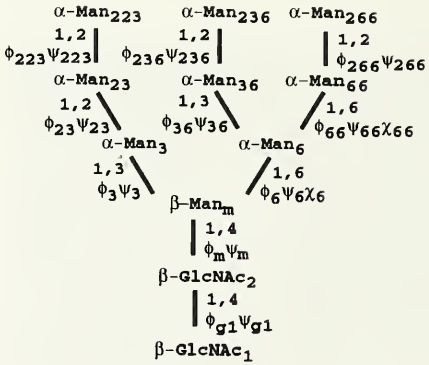
Several mannosidases differing in their subcellular localization, molecular and biochemical properties have been characterized by several research groups. No information is available about the size of the carbohydrate binding site of these α1,2-specific mannosidases. The present simulations show that the relative orientation of the Man-α1,2-Man disaccharide fragment with respect to the previous residue(s) in each antenna is different. If the binding site of the mannosidases accommodate more than the Man-α1,2-Man disaccharide fragment, it is likely that some of the α1,2-mannosidases may be highly specific to the terminal mannose residues on certain arms/branches. This perhaps explains the difference in the specificities of α1,2-specific mannosidases present in Golgi and ER.

Publications:

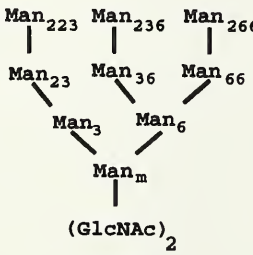
Balaji PV, Qasba PK, Rao VSR. Molecular dynamics simulation of asialoglycoprotein receptor ligands. Biochemistry 1993;32:12599-611.

Balaji PV, Qasba PK, Rao VSR. Molecular dynamics simulation of High Mannose Oligosaccharides. Glycobilogy, in press.

Figure 1
(Z01 CB 08397-03 LMMB)



G2M9



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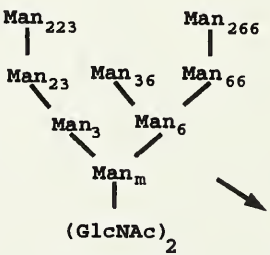
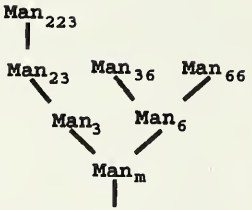
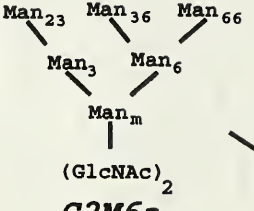


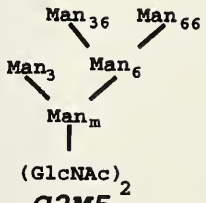
G2M8b



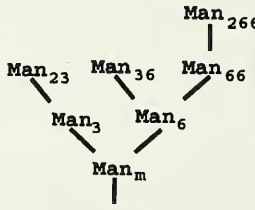
G2M7a



G2M6a



G2M5



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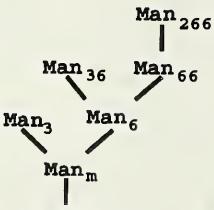

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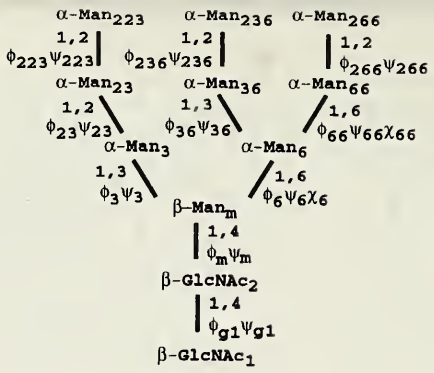
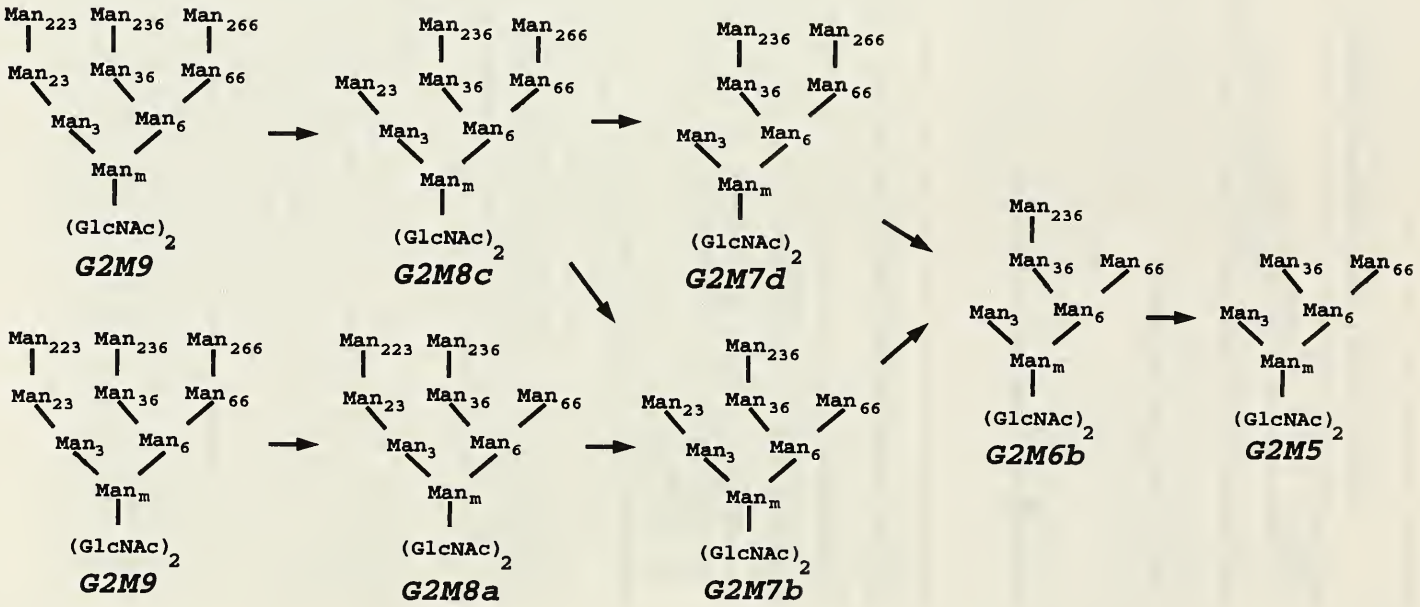


G2M9

Figure 2
(Z01 CB 08397-03 LMMB)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08399-02 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Functional Analysis of the Catalytic Domain of β1-4galactosyltransferase.

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

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:	PROFESSIONAL:	OTHER:
1.75	1.75	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The Golgi glycosyltransferases that are involved in the synthesis of oligosaccharides have a protein topology that consists of a short NH2-terminal cytoplasmic tail, a membrane signal anchor domain, a stem region and a COOH-terminal catalytic domain that binds a sugar-nucleotide and a sugar acceptor. The catalytic domain of β-1,4-galactosyltransferase (β-1,4-GT) transfers galactose from UDP-galactose to N-acetylglucosamine (NAGlc), either free or bound to an oligosaccharide, to produce N-acetyllactosamine with a β1-4 linkage.

Bovine β-1,4-GT and its NH2-terminal deleted and mutated proteins were expressed in *E.coli* as fusion proteins linked to the COOH-terminal end of glutathione-S-transferase (GST) protein. The recombinant fusion proteins were localized in inclusion bodies which were solubilized in 5 M guanidine-HCl. The conditions for renaturation and regeneration of the enzyme activity from the solubilized proteins were improved to give 80-90% renaturation efficiencies by changing the ratio of the reduced:oxidized glutathione from 8:1 mM to 10:1.25 mM and keeping temperature close to 40C during renaturation and subsequent dialysis. Analyses of the NH2-terminal deleted and substituted proteins have shown that the first 129 residues of the 402 residue long β-1-4GT are not involved in the enzymatic activity. NH2-terminal deletions of different lengths, including the first 129 residues of the protein, do not affect the Km's either for the sugar-nucleotide donor, UDP-galactose, or for the sugar acceptors, N-acetylglucosamine, chitobiose or chitotriose. Deletion or mutation of Cys134 resulted in complete loss of enzymatic activity. However, mutated Cys134 protein (Cys134Ala or Cys134Ser) or protein deleted up to residue 135 binds to UDP-agarose, N-acetylglucosamine-agarose and α-lactalbumin columns with the same efficiency as the non-deleted enzymatically active β-1,4-GT. These results suggest that Cys134 is involved in disulfide bond formation in the protein that is essential for enzymatic function.

PROJECT DESCRIPTION

Objective:

To determine: (a) the sugar-nucleotide donor and sugar-acceptor binding domains of β-1,4-galactosyltransferase protein 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 carboxy-terminal portion of the protein. The objective of the present studies is to determine from the NH_2-terminal deleted forms of β-1,4-GT protein produced in *E.coli*: (a) the kinetic constants for the sugar-nucleotide donor and sugar acceptors; (b) identify their binding domains; and (c) to produce the protein in large quantities for 3-D structure determination.

Major Findings:

Construction and expression of 5'-end deleted β-1,4-GT cDNAs and renaturation of the recombinant protein from the inclusion bodies.

The construction of 5'-end deleted forms of β-1,4-GT cDNA in pGEX-2T vector and expression of deleted forms of β-1,4-GT as fusion proteins (fused to the COOH-terminal end of glutathione-S-transferase) in *E.coli*, has been described in last years project number Z01 CB 08399-01 LMMB. The recombinant fusion proteins are present as insoluble inclusion bodies, which separate in the pellet fraction during the centrifugation of the lysed bacterial cultures. The insoluble inclusion bodies were dissolved in 5 M guanidine-HCl. The requirements and the conditions for the renaturation and regeneration of β-1,4GT activity, which is absolutely dependent on a mixture of reduced and oxidized glutathione, has also been described in last years project number Z01 CB 08399-01 LMMB.

Modifications in the renaturation conditions that increases the efficiency of renaturation and recovery of the fusion protein.

Previously, guanidine-HCl solubilized inclusion bodies were renatured in the presence of reduced and oxidized glutathione in a ratio of 8:1 mM, and the pH of the solution was 8.0. Using these conditions, the efficiency of renaturation and regeneration of the activity varied from experiment to experiment. Several modifications to these conditions were tested to increase the efficiency of renaturation and regeneration of the activity that would be reproducible. The following conditions were found to increase the efficiency of renaturation to approximately 90%. (1) The ratio of reduced and oxidized glutathione is to be 10:1.25 mM in the dilution buffer during renaturation step. (2) The renaturation buffer should be kept at 4^0C. (3) The guanidine-HCl solubilized protein should be added, dropwise, to the renaturation buffer that is constantly being stirred. (4) After dropwise addition of the solubilized protein to the ice cold renaturation buffer, the mixture should be gently stirred overnight at 4^0C. (5) Dialysis buffer should be ice cold and contain 150mM sodium chloride.

Enzymatic activity of deletion constructs and Cys134 mutants.

The fusion proteins GT-d75, GT-d119 and GT-d129, that lack NH_2-terminal 75, 119 and 129 residues, respectively, were active in β-1,4-GT and α-lactalbumin-dependent lactose synthetase (LS) activities. The fusion protein GT-d142, which lacks residues 1-142 that includes Cys134, and the mutant protein Cys134Ser, show neither β-1,4-GT 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 β-1,4-GT activity after thrombin treatment. The GST domain at

the NH_2-terminal end of the deletion construct, partially inhibits β-1,4-GT activity but not LS activity. Furthermore, in the presence of Mn^{++} both GT-d75 and GT-d129 proteins bind to UDP-Hexanolamine and GlcNAc-agarose columns, and can be eluted with 5 mM GlcNAc. Thus, residues 1-129 are not involved in the binding of either UDP-galactose or N-acetylglucosamine, but disruption of the disulfide bond involving Cys134 affects the enzymatic activity of the protein.

Kinetic constants of the NH_2-terminal deleted and mutated β-1,4-GT-fusion proteins.

The apparent K_m for UDP-galactose and sugar acceptors, N-acetylglucosamine (GlcNAc), chitobiose (GlcNAc-β-1,4-GlcNAc), chitotriose (GlcNAc-β-1,4-GlcNAc-β-1,4-GlcNAc) were measured for GT-d75, GT-d119, and GT-d129 fusion proteins and compared to the native milk bovine β-1,4-GT. The results showed that the NH_2-terminal deletions, up to residue 129, had no significant affect on the apparent K_m for the sugar-nucleotide donor and sugar acceptor substrates. Also, there was no affect on the V_{max} of the reaction.

Binding of deleted and mutated β-1,4-GT-fusion proteins to GlcNAc-agarose, UDP-agarose, α-lactalbumin and glutathione-sepharose columns.

In order to determine the efficiency of binding of deleted and Cys134 mutated β-1,4-GT-fusion proteins to UDP-, GlcNAc- and α-lactalbumin-agarose columns, GT-d129, GT-d142 and GT-d129C134S, proteins were produced as H^3 or S^{35} labeled proteins. During IPTG induction period, cultures were grown either in the presence of a mixture of H^3 labeled amino acids or in the presence of S^{35} labeled methionine. The labeled proteins were purified and renatured either alone or in the presence of H^3 labeled GT-d129 fusion protein, which upon renaturation generates an enzymatically active protein. Binding of the labeled renatured proteins to GlcNAc-agarose, UDP-agarose, α-lactalbumin and glutathione-sepharose columns, were measured either alone or in mixture with H^3 labeled GT-d129, which was used as an internal control to monitor the efficiency of renaturation. Either H^3- or S^{35}-labeled GT-d129 fusion protein bound 90% (± 5%), 80% (±5%), 73% (±5) and 35% (2%) to GlcNAc-agarose, UDP-agarose, α-lactalbumin and glutathione-sepharose columns, respectively. The β-1,4GT mutants with Cys134 deletion (GT-d142) or substitution (GT-d129C134S), also bound with almost the same efficiencies as H^3- or S^{35}-labeled GT-d129 fusion protein. These binding results suggest that the deletion or substitution of Cys134 β-1,4-GT does not affect the binding of the enzyme to the sugar-nucleotide donor or sugar acceptor substrate, but abrogates its enzymatic activity.

Publications:

Boeggeman EE, Balaji PV, Sethi N, Masibay AS, Qasba PK. Expression of deletion constructs of bovine β-1-4galactosyltransferase in *E. coli*: Importance of Cys 134 for its activity. Protein Eng 1993;6:779-85.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09300-01 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

MD Simulations of an Oligosaccharide in the lectin-carbohydrate crystals.

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

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NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

☐ (a1) Minors
☐ (a2) Interviews

B

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

Conformations of the heptasaccharide moiety of Erythrina corallodendron Lectin (EcorL), Man- α1,6-(Manα- 1,3)(Xyl-β1,2)-Man-β1,4-GlcNAc2-β1,4-(L-Fuc- α1,3)-GlcNAc1, the hexasaccharide Man- α1,6-(Man- α1,3)(GlcNAc-β-1,4)-Man-β1,4-GlcNAc-β1,4-GlcNAc and their disaccharide fragments have been studied by molecular dynamics (MD) simulations for 1000 ps with different initial conformations. These conformations were compared with the conformation of the heptasaccharide in the crystal structure of EcorL complexed with the oligosaccharide. In the isolated heptasaccharide the most frequently accessed conformation during MD has a ψ value of 180° around Man- α1,6-Man linkage. This conformation is stabilized by the formation of a hydrogen bond between the carbonyl oxygen of GlcNAc2 with the O3/O4 hydroxyls of the α1,6-linked mannose residue. The conformation of the heptasaccharide found in the crystal structure of the EcorL-lactose complex, that has a ψ value of about 76° around Man-α1,6-Man linkage, is accessed, though less frequently, during MD of the isolated oligosaccharide. The ϕ,ψ,χ = 58, -134°, -60° conformation around Man- α1,6-Man fragment observed in the crystal structure of the Lathyrus ochrus lectin complexed with a biantennary octasaccharide has also been accessed in the present MD simulations. These values for the α1,6 linkage, which are observed in the protein-carbohydrate crystal structures and are accessed in the MD simulations, though occasionally, have not been predicted from NMR studies. The less frequently accessed conformation of the heptasaccharide during MD simulations presents a better complementary surface to bind to the symmetry related lectin molecule in the crystal. If the heptasaccharide is not in this conformation while initiating the binding process, the conformation around Man6-α 1,6-Man_m will be altered by changing ψ to provide better complementary surface and to form additional hydrogen bonds with the protein at the expense of internal hydrogen bonds. Thus our results present a stereochemical explanation of the observed structures in the crystal of lectin-oligosaccharide complexes and also reveal the range of conformations an oligosaccharide can access, the information which is vital for understanding carbohydrate protein interactions.

PROJECT DESCRIPTION

Objective:

Molecular Dynamics (MD) simulations of oligosaccharides whose conformation in the crystal structure of lectin-carbohydrate complex is known.

Complex carbohydrates have been implicated in several biological functions even though their precise role is still not clear. Their initial interactions with proteins (or other macromolecules) is an a priori condition for the initiation of the biological response. Since these interactions occur with a unique conformer of the oligosaccharide, precise information about all the conformers which are accessible by the oligosaccharide are essential. Several conformers of an oligosaccharide exist in equilibrium in solution. As a result, most of the experimental studies in solution like NMR give a time averaged conformation which need not be the bioactive conformer. In view of this, molecular dynamics simulations are being increasingly used to obtain information about all the accessible conformations of oligosaccharides. The objective of the present study is to test if the MD simulation accesses the conformation of a heptasaccharide, whose conformation in the crystal structure of the lectin-carbohydrate complex is known, but was not predicted from NMR studies.

Recently, the crystal structure of *Erythrina corallodendron* Lectin (EcorL), a glycoprotein with an N-linked heptasaccharide Man-α1,6-(Man-α1,3)(Xyl-β-1,2)-Man-β-1,4-$GlcNA_c$-β1-,4-(L-Fuc-α1,3)-GlcNAc-β-1, has been reported (Shaanan *et al.*, 1991). The tertiary structure of the lectin in the subunit is similar to that of other legume lectins but has different quaternary structure because of the interference by the carbohydrate. Man-α1,6-Man in this heptasaccharide adopts a conformation (ϕ, ψ, χ = -62°, 76°, -67°) which is different from that deduced from solution studies or predicted from energy calculations on N-linked oligosaccharides without xylose. The three-dimensional structure of xylose containing N-linked heptasaccharide alone, has not been solved either by X-ray diffraction studies or analyzed completely by NMR. Also, no attempt has so far been made to analyze its possible conformations by MD simulations. Hence, it is not clear whether the low value of ψ (72° compared to 180° derived from NMR) for Man-α1,6-Man linkage in the heptasaccharide is due to xylose or protein induced conformational changes. Such information is important to the studies on the modulation of protein-protein interactions by carbohydrate moieties or in understanding the biosynthetic pathways of N-linked oligosaccharides. In the present study, molecular dynamics technique has been applied to study all the possible conformations of the heptasaccharide of EcorL (ChartI), and also of a hexasaccharide with bisecting GlcNAc on the trimannosidic core (Chart I) and their constituent disaccharide fragments. The conformational data obtained is compared with that from crystallographic and other studies.

Major Findings:

ϕ, ψ fluctuations of the constituent disaccharide fragments in the hepta- and hexa-saccharide.

The ϕ, ψ fluctuations of the disaccharide fragments, Fuc-α1,3-GlcNAc, GlcNAc-β-1,4-GlcNAc, Man-β-1,4-GlcNAc, Xyl-β-1,2-Man and Man-α1,3-Man in the haptasaccharide are highly restricted compared to their respective isolated disaccharides. In the bisected heaxasaccharide that lacks α1, 3-linked L-fucose (chart I), the ϕ, ψ fluctuations of the corresponding disaccharide fragments are also restricted compared to the respective isolated disaccharides, but more flexible compared to the heptasaccharide. The ϕ of Man-β-1,4-GlcNAc fragment in the heptasaccharide, accesses more frequently a value of 120^0 and less frequently negative values,

suggesting that L-fucose α1,3-linked to $GlcNAc_1$ reduces the flexibility of Man_m-β-1,4GlcNAc fragment in the heptasaccharide and its absence abrogates that effect in the hexasaccharide.

Man-α1,6-Man.

MD simulations were initiated with three initial values of χ, 180°; -60° and 60°. In all the simulations φ rapidly fluctuates around -55° and also goes as high as 60°. ψ has an average value of -170° but also accesses values around -60° and 60°. The fluctuations in χ are less compared to those in φ and ψ. However, χ favors all the three values 60°, 180°, -60° and spends longer durations of time before transition to another region. To observe the conformational transition in χ and thus obtain a reliable information about overall conformation, simulations can be performed either for several nanoseconds or several simulations of short duration with different initial values of χ can be done. Generally, -60° or 180° has been assigned for χ in related structures by NMR. A value of 60° for χ has not been reported from experiments. When χ assumes a value close to 60°, a hydrogen bond of the type O4-H...O(glycosidic) is favored. In the absence of this hydrogen bond this conformation is energetically less favorable than the other two because of electrostatic repulsion between O4 and glycosidic oxygen. The lack of observation of the χ = 60° conformation suggests that in aqueous media, the hydroxyl at the C4 may favor a hydrogen bond with solvent rather than with the glycosidic oxygen. Explicit placement of water molecules may reduce the preference for χ = 60° conformations.

The φ, ψ values of Man_6-α1,6-Man_m fragment in the heptasaccharide are also highly restricted compared to disaccharide, though they generally fluctuate around the same average value (-50°,180°). This change is more significant in the case of ψ. Unlike in a disaccharide, ψ_6 in the heptasaccharide accesses values close to 80° less frequently. χ_6 seems to access all three staggered conformations, though -60° is accessed predominantly. When χ is around -60° (with φ = -40°, ψ = 180°), the distance between carbonyl oxygen O7 of $GlcNAc_2$ and the O3/O4 hydroxyl hydrogens of Man_6 is less than 2 Å, suggesting that a hydrogen bond is possible between $GlcNAc_2$ and Man_6 residues. Such a hydrogen bond stabilizes the χ = -60° conformation and may explain the preference for this conformation in the heptasaccharide. The conformation with ϕ_6, ψ_6, χ_6 = 60°, -130°, -60° is accessed occasionally during MD simulations (40-90ps simulations in Fig. 6b). Such a conformation around the α1,6-linkage has been observed in the complex of the *Lathyrus ochrus* lectin with a biantennary N-acetyllactosamine octasaccharide, Gal-β-1,4-GlcNAc-β-1,2-Man-α1,6-(Gal-β-1, 4-GlcNAc-β-1,2-Man-α1,3-Man-β-1,4-GlcNAc by Bourne *et al.* (1992).

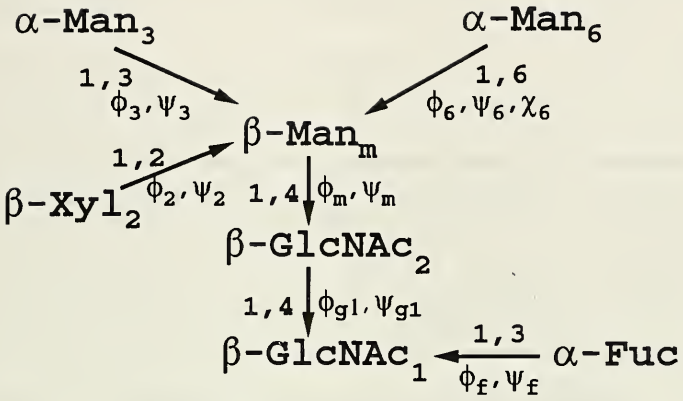
In the bisecting hexasaccharide ϕ_6, ψ_6 around Man_6-α1,6-Man_m linkage fluctuate mostly around -60° and 180°. ψ_6 also accesses occasionally, values around 60° and -120°. Such changes in ϕ_6 and ψ_6 (keeping χ_6 constant) brings in significant changes in the orientation of the α1, 6 arm, not only in bisecting hexasaccharide but also in heptasaccharide. In the bisecting hexasaccharide χ_6 accesses values around 180° and -60° and remains for longer durations of time before a transition to other conformations takes place. In this structure χ does not access values around 60° because of the bisecting GlcNAc. These results are in general agreement with the conclusions based on earlier NMR results by Homans *et al.* (1986). However, values of ψ around 70°, which are accessed rarely in these simulations, are not predicted from NMR studies. The conformational angles around Man-α1,3-Man fragment fluctuate around the same values (-40°,10°) in the disaccharide and in bisecting hexasaccharide and heptasaccharide which suggest that the presence of either bisecting GlcNAc or β-1,2-xylose may not affect significantly the orientation of α1,3-arm.

Comparison of the conformation of N-linked heptasaccharide in the crystal structure of EcorL-lactose complex with that derived from the MD simulations.

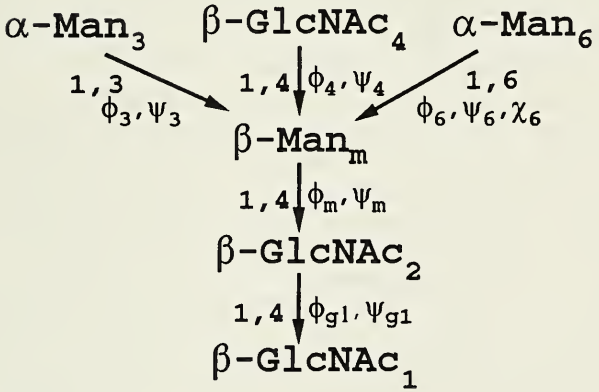
The crystal structure of the lactose complex of legume lectin (EcorL) with N-linked heptasaccharide was determined at 2.0A resolution to a R value of 0.19 by Shaanan *et al.* (1991). In the crystal, Asn-linked $GlcNAc_1$ and α1,3-linked L-fucose are held tightly by hydrogen bonds with side chain atoms of Asp16, Tyr53 and Lys55 of the same lectin molecule. $GlcNAc_2$, the trimannosidic core and xylose form nine hydrogen bonds with the main chain and side chain atoms at the β turns of the neighboring lectin molecule. The conformation of the disaccharide fragments, GlcNAc-β-1,4-GlcNAc (ϕ, ψ = 47°, 11°), Man-α1,3-Man (ϕ, ψ = -34°, 5°), Xyl-β-1,2-Man (ϕ, ψ = 45°, 19°) and L-Fuc-α1,3-GlcNAc (ϕ, ψ = 45°, 19°) of the heptasaccharide found in the crystal structure are accessed frequently in the MD simulations. Although the conformation (ϕ, ψ = 41°, -37°) of the Man-β-1,4-GlcNAc fragment is accessed in the MD simulations, ψ deviates from the average conformation. The conformation (ϕ, ψ, χ = -62°, 76°, -67°) for Man_6-α1,6-Man_m fragment observed in the crystal is accessed very frequently in the MD simulation of the isolated disaccharide but rarely in the heptasaccharide. One such rare conformation accessed by the heptasaccharide can been superposed over that observed in the crystal structure of EcorL. These results suggest that the Man_6-α1,6-Man_m linkage adopts a less favorable conformation in the crystal and the less frequent conformations of the heptasaccharide present a better complementary surface to bind to the symmetry related lectin molecule in the crystal. If the heptasaccharide is not in this conformation while initiating the binding process, the conformation around Man_6-α1,6-Man_m will be altered by changing ψ from its preferred value of 180° to 76°, to provide better complementary surface and to form additional hydrogen bonds with the protein at the expense of internal hydrogen bonds (Man_6-O3/O4 to $GlcNAc_2$-O7). It should be noted that such a conformation (-62°, 76°, -67°) for Man_6-α1,6-Man_m has not been considered while proposing probable conformations for N-linked oligosaccharides from NMR.

Influence on the orientation of α1,6-arm by changing ψ_6 instead of χ_6.

From the present studies, regarding α1,6-arm, three important conclusions can be drawn: (1) The orientation of the α1,6-arm, as previously noted by Brisson & Carver (1983) and Biswas *et al.* (1986), is affected by changes in χ. When χ_6 is around -60° the trimannosidic core is more open than in the conformation where χ_6 is around 180°. (2) The orientation of the α1, 6-arm can also be affected significantly by the changes in ϕ and ψ, keeping χ constant. At constant χ the orientation of α1,6-arm changes as ϕ and ψ changes resulting in an overall change in the shape of the molecule. (3) χ_6 being either -60° or 180° does not influence the orientation of the α1,3-arm neither in heptasaccharide (with β1,2-Xyl) nor in GlcNAc bisecting hexasaccharide. From these results one can suggest that the differences in the biological actvities of N-linked oligosaccharides can't always be ascribed to the differences in the orientation of α1,6-arm brought about by merely changing the two possible values of χ (-60° and 180°), nor can the changes in biological activities be attributed to the changes in the orientation of α1,3-arm as previously suggested by Homans *et al.* (1987). The present simulations also show that the addition of bisected GlcNAc to the trimannosidic core of the hexasaccharide does not change significantly the orientation of the α1, 3-arm or α1,6-arm. The bisecting GlcNAc on the trimannosidic core may exercise control on the bisynthetic pathways not by changing the orientation of either the α1,3- or the α1,6-arm but by stericaly interfering with the binding sites of transferases.



Heptasaccharide



Bisected Hexasaccharide

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09301-01 LMMB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

HIV-1 Infection

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

Dimiter S. Dimitrov, Ph.D. Visiting Scientist MS&FS, LMMB, NCI

Other Professional Personnel:

Robert Blumenthal, Ph.D. Section Chief MS&FS, LMMB, NCI

COOPERATING UNITS *(if any)*

Dr. H. Golding, CBER; Dr. M. Martin, Dr. C. Broder and G. Englund, NIAID; Dr. L. Burkly, Biogen; Dr. S. Dusing, Quality Biological, Inc.; Dr. R.M. Anderson, University of Oxford, UK.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure and Functions Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The major goal of this project has been to quantify stages of the HIV-1 life cycle, and by using a variety of biophysical, cellular and molecular biology techniques combined with mathematical modeling to understand how replication kinetics is affected by viral and cellular structures. A novel approach for quantitation of HIV-1 infection kinetics has been developed which allows the determination of a critical parameter of a spreading virus infection, the number of infecting virions produced by an infected cell and transmitted to uninfected cells, as well as the time required for one cycle of infection. Based on this and other approaches, it was found that: (1) HIV-1 infection of peripheral blood mononuclear cells (PBMC) and macrophages required about the same time to accomplish one cycle (3-4 days), while the number of infecting virions produced by one infected PBMC was 10-fold higher (about 100) than from a macrophage. The cell-to-cell transmission of HIV-1 was much more efficient (102-103-fold) than by cell-free virus; (2) Phorbol esters modulated an HIV-1 co-receptor molecule(s). This is not CD26, which has been recently suggested as a CD4 co-receptor (Science 262:2045-2050); (3) The 3' end of the HIV-1 U5 region is not required for reverse transcription as has been suggested for other retroviruses (e.g., Moloney murine leukemia virus). An analysis of the HIV-1 infection kinetics, in combination with other data, indicates that the 3' end of the U5 region has some additional unknown functions related to integration; (4) An antibody against the second domain of CD4 synergistically inhibited HIV-1 infection and syncytia formation when combined with antibodies against the HIV-1 envelope glycoprotein; (5) gp41 of the HIV-1 envelope glycoprotein has cytopathic effects when expressed by recombinant vaccinia virus under the control of a strong promoter; and (6) The CD4 cell number of some HIV-1 infected individuals declines with time following a two-exponential law.

These findings have implications for understanding the mechanisms controlling the HIV-1 life-cycle and the development of AIDS, and for a rationale design of antiviral drugs.

PROJECT DESCRIPTION

1. Kinetics of HIV-1 infection in PBMCs and macrophages and its neutralization

We have recently developed a novel approach to analyze HIV-1 infection kinetics in tissue culture systems (J Virol **67**:2182-90) and have used it: (1) to quantitate the number of infecting virions transmitted from infected to uninfected cells and (2) to determine the time needed for one cycle of infection. This approach has now been applied to infection of macrophages and peripheral blood lymphocytes (PBMCs) by unpassaged primary HIV-1 isolates. While the number of infecting virions transmitted to uninfected PBMCs was very similar to that for laboratory adapted strains in T cell lines (about 100 per cell per cycle), it was 10-fold lower for a spreading infection in macrophages. Interestingly, the time required to complete one cycle of infection was approximately the same for PBMCs, macrophages and T cell lines (about 3 days). Infected PBMCs or infected macrophages transmitted HIV-1 to uninfected macrophages or PBMCs, respectively, with very high efficiency. These results demonstrate that the infection kinetics in macrophages is slower than in PBMCs because of the lower number of infecting virions transmitted to uninfected cells rather than changes in the rate of virus replication. The infection kinetics approach has also been applied to the antibody mediated neutralization of HIV-1 in which the effects of serum samples from seropositive individuals on the infectivity of different virus preparations (including an unpassaged recent HIV-1 isolate [DH012] kindly provided by M.D. Hoggan, NIAID, NIH) in peripheral blood lymphocytes (PBMCs) has been analyzed. HIV-1 was preincubated with sera (kindly provided by L. Vujcic, CBER, FDA) and then mixed with PBMCs or monocyte derived macrophages. Infection kinetics was measured by a reverse transcriptase assay and the reduction of virus infectivity determined by measuring the times needed to reach the peaks of virus production. Undiluted patients' sera reduced the infectivity of DH012 for PBMCs 10-fold; a 10-fold dilution of sera reduced its infectivity by 50%. The neutralizing titers of the sera for tissue culture passaged isolates of HIV-1 such as IIIB, RF, MN, SF2 and Z3 as previously determined (L. Vujcic, unpublished) were 512/1024, 16/64, 12800, 2048 and 64. These results demonstrate that unpassaged HIV-1 isolates are neutralized by sera but at lower efficiency and that quantitation of infection kinetics can be used to evaluate their neutralization.

2. The HIV-1 co-receptor molecule(s) is modulated by phorbol esters

The phorbol ester PMA strongly inhibits HIV-1-induced syncytium formation; it has been suggested that this inhibitory effect is due to the transient downmodulation of the surface associated CD4 receptors by PMA (Virol 176:126-32). Surprisingly, PMA treatment of cells expressing truncated (A2.01.CD4.401) and hybrid (A2.01.CD4.CD8) CD4 molecules, which are not downmodulated (Nature 334:162-5), inhibited their fusion with $CD4^-$ (12E1) cells expressing vaccinia encoded HIV-1 envelope glycoprotein (gp120-gp41) and with chronically HIV-1 infected H9 (MN, IIIB, or RF) cells. PMA pretreatment of T (12E1) and non-T (HeLa, U937.3 and EBV-transformed B) cell lines expressing vaccinia encoded CD4 also blocked fusion with 12E1 cells expressing vaccinia encoded gp120-gp41. Interestingly, pretreatment of the gp120-gp41-expressing 12E1 cells with PMA did not alter their fusion with untreated CD4-expressing cells. While the inhibitory effect of PMA was rapid and treatment for 1.5 h with 5 ng/ml PMA was sufficient to reduce fusion by more than 50%, the recovery after treatment was slow and more than 40 h were needed before the cells restored half of their fusion potential. The PMA inhibitory effect was blocked by staurosporine in a dose dependent fashion, suggesting that it is mediated by protein kinase C. PMA treatment of A2.01.CD4.401 cells reduced the number of infected cells 6.7-fold, as estimated by a quantitative analysis of the HIV-1 (MN) infection kinetics, probably by affecting the stage of virus entry into cells. CD26 surface expression was not

significantly changed by PMA treatment. We conclude that PMA inhibits the CD4-gp120-gp41-mediated fusion by modulating accessory component(s), different than CD26, in the target CD4 expressing cells. These findings suggest a novel approach for identification of CD4 co-receptor molecules and may have implications for development of antiviral agents.

3. The 3' end of the HIV-1 U5 region is not required for reverse transcription

A PCR-based approach has been developed, in combination with a video imaging system, allowed accurate quantitation of viral DNA synthesis following infection of T lymphocytes with HIV-1 U5 deletion mutants. The kinetics of accumulation of viral DNA in infected cells was measured for the wild-type HIV-1 NL4-3, a mutant with a 26 bp deletion at the 3' end of U5 (excluding the 4 bps at the very 3' end of U5 which are important for integration) and a revertant where the original 26 bp deletion was further extended upstream to a total of 45 bp deletion. The viral DNA increased to a maximum of 5 molecules per infected cell 11 hours after the start of infection and then decreased to 3 molecules per infected cells 13 hours later. There was no significant difference (less than 2-fold) between the levels of wild type, mutant and revertant DNA throughout the 24-hour infection kinetics experiment. An infection kinetics analysis of the virus spread in tissue culture suggested that the number of wild-type viral DNA molecules should have been 20-fold and 5-fold larger than that of the mutant and revertant, in order to explain the experimentally measured HIV-1 spreading kinetics. Interestingly, *in vitro* experiments (performed by A. Engelman) showed the same ratios for the integration efficiency of the wild-type HIV-1 to the mutant and revertant viruses. Therefore, the 3' end of U5 is not required for reverse transcription but rather is related to integration by an unknown mechanism. The results also suggest that the integration efficiency *in vivo* is relatively high because at least 1 out of 5 molecules viral DNA becomes integrated and leads to productive infection.

4. A monoclonal antibody (5A8), against the second domain of CD4, inhibits synergistically, HIV-1 infection and syncytia formation when combined with anti-gp120 antibodies

Previously, it has been found that a CD4 domain 2-specific monoclonal antibody, 5A8, inhibits HIV-1 entry without interfering with gp120 binding to CD4, presumably by affecting a post-binding membrane fusion event. Because antibodies to the gp120 V3 loop also affect post-CD4-gp120 binding events, 5A8 was tested in combination with anti-V3 loop antibodies for possible synergy. The anti-V3 loop antibodies, 0.5 β and NEA9205, synergistically inhibited HIV-1IIIB infection and syncytia formation between chronically infected H9 cells and C8166 cells. Another anti-V3 loop antibody, 110.5, inhibited also synergistically syncytia formation between cells expressing gp120-gp41 and Molt-3 cells. In contrast, a human monoclonal antibody to an epitope of gp120 involved in CD4 binding, IAM 120-1B1, exerted only an additive effect in combination with 5A8. Sera from HIV-1 infected individuals, which presumably contains antibodies to the V3 loop and the CD4 binding site, synergistically inhibited HIV-1 infection and syncytia formation. These results demonstrate that therapeutics based on antibodies affecting non-gp120-binding epitopes of the target receptor molecule CD4 would be more efficient in patients with anti-V3 loop antibodies.

5. The gp41 glycoprotein of HIV-1 is cytopathic

We have constructed a recombinant vaccinia virus where a major part of gp120 is deleted and gp41 is expressed under the control of a very strong synthetic vaccinia promoter. The expression of large amounts of gp41 led to high cytopathic effect. We are continuing our investigation for other possible effects of gp41 expression.

6. Mathematical modeling of CD4 cell number in HIV-1 infected individuals

CD4 T cells commonly decline with time in HIV-1 infected individuals. Several mathematical models have been developed to describe that decline and possibly elucidate the factors which are responsible for it. We have found, however, that in several cases the equations used to describe the HIV-1 infection *in vivo* are not applicable even to the much more simple case of HIV-1 infection in tissue cultures. Therefore, we tried to describe the existing data for the CD4 cell decline by empirical formulae. Interestingly, we found that for a subset of HIV-1 infected individuals participating in the MACS the CD4 cell decline can be approximated by a two-exponential function. In several cases the data can be described even with one exponent. Several models, based on different assumptions, can result in such exponential decline. Presently, we are trying to find quantitative data in the literature and/or design appropriate experiments which could allow us to discriminate between alternative models.

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SUMMARY STATEMENT

LABORATORY OF MOLECULAR BIOLOGY

DCBDC, NCI

OCTOBER 1, 1993 to SEPTEMBER 30, 1994

The Laboratory of Molecular Biology uses genetics, molecular biology, and cell biology to study gene activity and cell behavior. One major goal is to develop new approaches to the treatment and diagnosis of cancer, AIDS and other human diseases. Another is to understand how cell division and gene activity is regulated.

Immunotoxin and Recombinant Toxin Therapy of Cancer:

For the treatment of human cancer and AIDS, I Pastan and colleagues have designed and produced both conventional immunotoxins and fully recombinant toxins. Immunotoxin LMB-1 (a conventional immunotoxin composed of MAb B3 and a mutant form of *Pseudomonas* exotoxin, PE) is in clinical trials on patients with colon, breast, stomach and esophogeal cancers. LMB-7 is a smaller recombinant immunotoxin also made with the B3 antibody. Its small size should improve tumor penetration. LMB-7 will enter trials in the same patient population in 1994. Other immunotoxins directed at the erbB2 oncoprotein (e23Fv-PE38KDEL) and at the IL2 receptor (anti-Tac(Fv)PE38KDEL) present on lymphomas and leukemias will enter the clinic in 1995. TP40 (TGFα fused to a 40,000 MW fragment of PE) is being evaluated in patients with bladder cancer. A very active recombinant toxin directed at the IL4 receptor present in kidney cancers has recently been made.

Recombinant immunotoxins with improved properties have been made which are: (1) smaller and therefore penetrate into tumors better; (2) less immunogenic and (3) more stable and easier to produce. Increased stability was achieved by using a combination of molecular modeling and genetic engineering to insert a disulfide bond at a conserved location in the framework region.

Site directed mutagenesis has been used to determine which surface residues in domains II and III of PE can be changed to cysteine (or other amino acids) without loss of activity; several sites were identified. These cysteine residues were then used to make specific polyethylene glycol (PEG) derivatives of PE. Even though large PEG molecules were added to PE, almost full cytotoxicity was maintained.

Previously it was found that a large portion of domain Ib of PE could be deleted without loss of activity. Pastan and colleagues have recently found that all of domain Ib (aa 365-395) and a portion of domain II can be deleted from single chain immunotoxins, if a glycine-rich connecting peptide is inserted between the remainder of domain II and domain III to keep the domains separated.

Monoclonal Antibodies to Cancer Cells:

To deliver toxins or other therapeutic agents to cancer cells, it is necessary to have antibodies that selectively recognize these cells. One approach to this problem is to identify differentiation antigens that are present on nonessential tissues and continue to be expressed on cancers derived from them. K. Chang and I. Pastan have isolated two monoclonal antibodies (K1 and PR1) that have these properties. MAb K1 reacts with a differentiation antigen present on the surface of normal ovary and on most ovarian cancers. The antigen is a 40 kDa glycosylated protein linked to the cell surface by phosphatidyl inositol. To clone the antigen, K. Chang has screened expression libraries made from ovarian cancers and ovarian cancer cell lines. Initially, a cDNA encoding a 34 kDa protein was isolated and sequenced. This antigen is a cytosolic protein that is abundant in ovarian cancer cells but is not the CAK1 antigen. Recently a new cDNA clone was isolated which encodes a protein with properties expected for the antigen present on the surface of ovarian cancer cells. Further sequencing and epitope mapping are underway to establish its identity.

Monoclonal PR1 is an IgM that reacts with the surface of normal prostate, almost all primary prostate carcinomas, and many metastatic prostate cancers. It is expressed in very few other normal tissues or non-prostate tumors. K. Chang has identified a protein which reacts with the PR1 antibody. It is a 34 kDa protein found in the membrane fraction of LNCaP cells and its levels are increased by growth of these cells on laminin. By screening an expression library made from human prostate, several clones were obtained that react with the PR1 antibody. One of these has the sequence of the laminin binding protein isolated by M. Sobel (NCI) and coworkers. Other clones with different sequences have also been isolated. The relationship of these cDNA clones to the PR1 antigen is under investigation.

Development of Immunotoxins for Cancer:

FitzGerald and colleagues study how PE enters cells and is processed to the form needed for translocation. *Pseudomonas* exotoxin uses the alpha 2 macroglobulin receptor (also called LRP) to enter cells and inhibt protein synthesis. Mutant cells that expressed reduced amounts of LRP were toxin resistant. Resistance was an LRP-specific defect since mutants retained their sensitivity to a conjugate of transferrin chemically linked to PE40. Once inside cells, PE is cleaved by a cellular protease. This cleavage, which is essential for generating a cytotoxic fragment from the C-terminal portion of PE, was shown to be mediated by a furin-like protease. The biological relevance of furin cleavage was confirmed by showing that nicked toxin killed cells more rapidly than uncleaved toxin. A similar result was obtained with diphtheria toxin (DT). Comparing the processing of the two toxins, it was shown that PE was cleaved optimally at pH 5.0-5.5 while DT was cleaved best at pH 8.0. In addition, the kinetics of PE cleavage were slow compared to that of DT. The furin-mediated cleavage of the chimeric toxin TGFα-PE38 was also investigated. Results suggest that cleavage occurs at the same site and proceeds with the same characteristics as with native PE. In an effort to kill myelogenous leukemia cells, PE38 was chemically coupled to a monoclonal antibody directed to the CD33 antigen.

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells:

The work of Pastan and Gottesman emphasizes two aspects of the molecular biology of multidrug resistance (MDR) in cancer cells: 1) the mechanism of action of the multidrug transporter also known as P-glycoprotein (PGP), the product of the *MDR*1 gene; and

2) the use of vectors encoding the *MDR*1 gene to confer selective advantage on cultured cells and in cells in intact animals and patients. Mechanistic studies have involved biochemical purification of both naturally occurring (from MDR cell lines) and recombinant (from baculovirus) PGP. PGP purified to near homogeneity demonstrates drug-dependent ATPase activity and transport activity when reconstituted in lipid vesicles. Phosphorylation of PGP is not necessary for its activity, as shown by recombinant expression vectors in which all of the known phosphorylation sites on PGP have been deleted. A new kinase present in plasma membranes of cultured cells which phosphorylates PGP has been discovered and purified from cultured cells. To develop a model system for studying the effect of specific mutations on ATPase and substrate binding activities of PGP, the human *MDR*1 cDNA has been expressed in the yeast Saccharomyces cerevisiae, resulting in resistance to several different anti-cancer drugs. For gene therapy of cancers, *MDR*1-encoding retroviral vectors which confer MDR on the bone marrow of mice have been developed. One bicistronic vector in which the Herpes simplex virus thymidine kinase gene is under control of an internal ribosome entry site (IRES) present on the same mRNA which encodes *MDR*1 can be used to kill cancer cells which have been inadvertently transduced with the *MDR*1 gene during gene therapy to protect bone marrow against the toxic effects of chemotherapy. Other vectors for gene therapy of inborn errors of metabolism carry the *MDR*1 cDNA as a selectable marker and the non-selectable cDNAs for glucocerebrosidase (Gaucher disease), alpha galactosidase (Fabry disease) and the 91 kDa subunit of the NADPH oxidase complex (chronic granulomatous disease).

Mechanisms of Thyroid Hormone Action in Animal Cells:

Thyroid hormone receptors (TRs) are ligand-dependent transcription factors which regulate growth, differentiation and development. The gene regulating activity of TRs is affected by the hormone, 3,3',5,-triiodo-L-thyronine (T_3), dimerization with other nuclear factors and the specific DNA element in the promoter region of T_3 target genes (hormone response element, TRE). To understand the molecular mechanism by which T_3, dimerization and TRE modulate the gene regulating activity of TR, S.-y. Cheng and colleagues have studied (i) the structure of the hormone binding domain and hormone binding site with the ultimate aim of understanding how the hormonal signal is transduced from the hormone binding domain to the DNA binding domain of TR to affect the interaction of receptor with TRE; (ii) the effect of phosphorylation of TR on heterodimerization and on TRE/receptor interaction. Their results suggest that the hormonal signal that affects the gene regulating activity of human β1 TR (h-TRβ1) is most likely transmitted by T_3-induced conformational changes. Based on the proposed 8-stranded α/β barrel structure for the hormone binding domain of h-TRβ1, they found that the regions of the hormone binding domain which undergo conformational changes are near the DNA binding domain, thereby affecting the interaction of TR with DNA.

They also found that phosphorylation is essential for h-TRβ1 to heterodimerize with the retinoid X receptor (RXR). Phosphorylation not only increases the binding of h-TRβ1 to RXR on TREs, but also augments the RXR-dependent h-TRβ1 transcriptional activity. However, the degree of enhancement induced by phosphorylation is TRE-dependent.

The information derived from the above studies should be valuable in understanding the pathogenesis of a human disease, generalized resistance to thyroid hormone (GRTH). Cheng and colleagues found patients with GTRH have mutations in the hormone binding domain of h-TRβ1. As a result, the T_3 binding activity is reduced and dimerization and TRE binding are also altered.

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

Merlino and coworkers study the role of peptide growth factors and differentiation factors in normal and disease processes using transgenic mice. They have already established several transgenic animal models of human diseases, including cancer. Over the past year, they have continued their analysis of transgenic mice overexpressing transforming growth factor α (TGFα), which has been implicated in human cancer. They have shown that TGFα transgenic mice develop a high incidence of liver and mammary tumors, as well as premalignant hyperproliferative lesions of the pancreas and stomach. They have shown that TGFα overexpression induces malignant disease in other mouse tissues, but only in collaboration with other endogenous oncogenes and factors. Another molecule, transforming growth factor β (TGFβ), possesses a wide variety of activities that often oppose those of TGFα. They have generated TGFβ transgenic mice which are deficient in mammary gland development and milk production. Importantly, TGFβ was found to inhibit the formation of TGFα-induced mammary tumors in their TGFβ/TGFα bitransgenic mice. These and other related transgenic mice serve as useful molecular models for studying the cause and development treatment of important human diseases.

Regulation of Gene Activity:

A. Johnson's studies regulation of the epidermal growth factor receptor (EGFR) gene and how alterations in its regulation lead to cancer. The EGFR is overproduced in several types of cancer and overexpression can lead to EGF-dependent transformation. In some cancers, overproduction of the EGFR gene is due to gene amplification. In others, such as breast, ovarian, cervical and kidney tumors, over-expression of EGFR results from transcriptional or posttranscriptional mechanisms. To understand how overexpression of the EGFR gene occurs in these tumors, he has been defining the DNA and protein elements that are involved in the expression of the EGFR gene. The regulatory region of the gene has been isolated and sites to which regulatory proteins isolated from nuclear extracts bind have been delineated. Nuclear proteins that act to increase EGFR gene expression or to decrease expression have been identified. Using a cDNA clone for a repressor of transcription termed GCF, he examined the expression of GCF in a variety of cultured cell lines. Also antibodies to GCF were generated and used to show that GCF is primarily a nuclear phosphoprotein. He has isolated a partial cDNA clone that contains homology to the GCF DNA binding domain and examined expression of this gene in cancer cell lines. He also localized the gene for this cDNA to the chromosome 20q13.3 region that has been shown to be amplified in some breast cancer cell lines. He has also participated in the identification of a new regulator of EGFR gene expression found in extracts from A431 and HeLa cells.

Genetic Regulatory Mechanisms in *Escherichia coli* and its Bacteriophage:

The Developmental Genetics Section of the Laboratory of Molecular Biology conducts basic research in understanding molecular mechanisms of gene expression at different levels. S. Garges and S. Adhya are studying the mechanisms of positive control of gene transcription by cAMP receptor protein (CRP), in the *lac* promoter of *Escherichia coli*. A number of experiments from their laboratory and other laboratories have provided evidence suggesting that activation of transcription initiation requires a protein-protein contact between DNA-bound cAMP•CRP complex and RNA polymerase. Garges and Adhya have now obtained evidence that shows that transcription activation mediated by DNA-bound cAMP•CRP also requires a structural change in the DNA. They have proposed that a cAMP•CRP induces a structural change in DNA that is transmitted to the promoter.

Distortion in the double helical polynucleotide structure either by creating a two to ten base gap in one of the two strands or by the use of mismatched base-pairs in the segment of DNA between the cAMP•CRP binding site and the promoter inhibits transcription activation.

Regulation of the *gal* Regulon of *Escherichia coli*:

S. Adhya is also studying negative control of transcription in the gene involved in the transport and metabolism of D-galactose in *Escherichi coli*. The *gal* regulon contains at least five operons, two of which encode for the repressors, GalR and GalS. The *gal* operon, a typical member of the regulon, is transcribed from two promoters, P1 and P2, located on opposite faces of the DNA helix. Complete repression of transcription of P1 and P2 requires looping of the promoter DNA segment which occurs because of interaction of repressors bound to two operator sites, O_E and O_I, which encompass P1 and P2. Adhya and his group have also shown that if the DNA loop formation is prevented, occupation of the O_E site alone by a repressor molecule is sufficient to repress P1, and not P2, at an 80% level. Based on these and other genetic and biochemical experiments, they have proposed that repressor bound to O_E inhibits transcription initiation from P1 by a direct contact with the promoter bound RNA polymerase. As expected from this model, they have also found that RNA polymerase binding to the *gal* promoter is not inhibited by the binding of repressors to O_E and/or O_I DNA elements.

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

D. Jin is studying the underlying biochemical mechanism and regulation of the three steps of transcription initiation in *Escherichia coli*: (1) RNA polymerase binding to the promoter; (2) isomerization of the enzyme-DNA complex (formation of a heparin resistant complex); and (3) clearance from the promoter (movement of RNA polymerase from its initial binding site accompanied by formation of RNA oligomers). By a combination of genetics and biochemistry, he has found that in two different promoters, the *pyrBI* promoter and *galP2* promoter, RNA polymerase stutters, without leaving the promoter, at a short stretch of adenine residues present in the template DNA and makes nonproductive RNA with long stretches of uridines. Moreover, such pseudo-templated RNA synthesis occurs only when the UTP concentrations are high. The biological significance of such observations in the *pyrBI* and *galP2* promoters and not at other promoters is understood by the fact that the *pyrBI* and *gal* operons are involved in the biosynthesis of UTP and UDP-sugar derivatives. By the stuttering mechanism, productive transcription of these operons is suppressed when the end products are present in excess.

Bacterial Functions Involved in Cell Growth Control:

Susan Gottesman and colleagues study the method by which energy-dependent proteases are able to select specific unstable substrates out of a sea of stable intracellular proteins. In *E. coli*, both the protease and targets are amenable to genetic manipulations to define essential regions. The Lon ATP-dependent protease appears to use signals throughout its target for recognition. ClpAP protease recognition depends on the ClpA ATPase subunit and specific interactions of ClpA with the ClpP proteolytic subunit. Targets for proteolysis are frequently subject to multiple levels of control, exemplified by the finding of a chromatin-like silencing of the promoter for the gene encoding RcsA, a Lon target;

silencing is overcome by a unique small RNA. Coupling of cell division to chromosome partition is also being studied through the analysis of mutants in the cell cycle gene *mbrA*.

Molecular Chaperones and DNA Replication:

S. Wickner and D. Skowyra study the mechanism of action of molecular chaperones and their role in DNA replication. They found that three *E. coli* heat shock proteins, DnaJ, DnaK (the Hsp70 homologue), and GrpE are required for plasmid P1 DNA replication *in vitro*. and discovered 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 with high affinity to *ori*P1 DNA. GrpE 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 μM by a metal ion buffer system. GrpE specifically lowers the concentration of Mg^{+2} required for ATP hydrolysis by DnaK, suggesting that this may be the step in RepA activation where Mg^{+2} is essential. They also discovered that ClpA, the regulatory component of the ATP-dependent ClpAP protease, activates the sequence specific DNA binding activity of RepA. ClpA forms an ATP-dependent complex with RepA and converts RepA dimers to monomers with ATP hydrolysis. ClpA also acts as a chaperone in protecting luciferase from irreversible heat inactivation but unlike DnaJ, DnaK and GrpE it is unable to reactivate the inactivated luciferase. These results demonstrate that ClpA is a new molecular chaperone.

Molecular Modeling:

The Molecular Modeling Section (B. K. Lee and colleagues) uses and develops theoretical tools with which to study the forces that govern the structure and interaction of globular protein molecules, to study and predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties. In the past year, they: (1) clarified the relation between the enthalpy-entropy compensation phenomenon and hydrophobicity, (2) found additional evidence that size of water molecules is the overriding factor in determining the hydrophobicity, (3) obtained a 3 Å structure for the crambin molecule by guiding the folding process, (4) devised and tested a simplified but rapid way of estimating the hydrophobic energy to be used in protein folding studies, and (5) devised a structural alignment scheme tailored to the α/β barrel structures and found evidence of sequence shuffling in some members of this family of proteins. They also studied hybrid molecules of the *Pseudomonas* exotoxin (PE) with different antibody Fv's, with interleukin 4, and with TGFα and made a number of recommendations to improve various properties of these molecules by site-directed mutagenesis. Some of these have been tested and found to have the desired property. The most important of these was the design of a Fv fragment of an antibody that is stabilized by a disulfide bond that connects the framework residues.

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

PROJECT NUMBER
Z01 CB 08000-24 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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	Expert	LMB, NCI
Other:	E. Piebenga	Undergraduate student	Colgate University

COOPERATING UNITS (if any)
X. Hou and M. R. Rosner, The Ben May Institute and Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Biology

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	0.25

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

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

The expression of the epidermal growth factor receptor (EGFR) gene is strictly regulated to ensure proper cell growth. Overexpression of the EGFR gene has been detected in many tumors. To study the regulation of this gene, we have previously isolated the promoter region and also identified some of the transcription factors acting as positive and negative regulators. We have previously utilized a cloned cDNA and antiserum to determine that GCF is a transcriptional repressor of the EGFR gene. We have shown that GCF is a phosphoprotein found primarily in the nucleus and is more abundant in cell with lower numbers of EGFR. We have also determined that the 3.0 kb mRNA encoding GCF is expressed at low levels in breast cancer cell lines that have either high or low numbers of EGFR per cell.

The GCF cDNA hybridizes to two additional mRNA species of 4.5 and 1.2 kb. A cDNA that encodes most of the 4.5 kb mRNA has been isolated and sequenced. The deduced sequence of the protein encoded by the 4.5 mRNA showing it is rich in glutamic acid residues and has a pI of 4.3. The molecular weight is at least 78,000. This protein would contain a basic region similar to the DNA binding region of GCF. The gene for this product has been localized to the chromosome 20q13.3.

In addition to GCF, we have identified another transcriptional repressor located 5' to the GCF binding sites. EGFR transcriptional repressor (ETR) binds to a TTCGAGGG sequence at -877 to -870 in the EGFR promoter. A fragment from the EGFR promoter containing this region acts as a repressor when linked to the thymidine kinase promoter. Crosslinking studies have shown that the protein binding to this region has a molecular weight of 128,000 and is present in extracts from HeLa and KB cells.

We have isolated three P1 clones containing genomic sequence for GCF.

Major Findings:

The regulation of the EGFR gene is very complex and involves interaction of the promoter region with activators and repressors. GCF is a transcriptional repressor of the EGFR gene. We have shown that GCF is primarily localized to the nucleus and is phosphorylated on serine and threonine residues. An antiserum to GCF was used to show that GCF is expressed at high levels in cell lines with low EGFR expression.

GCF is encoded by a 3.0 kb mRNA . This mRNA is present in eight breast cancer cell lines that were examined by northern blot hybridization analysis. There was no clear correlation of GCF mRNA level and EGFR level in these cell lines. Two of these breast cancer cell lines (MDA-MB-231 and MDA-MB-468) expressed two- to four-fold higher levels of GCF mRNA. Both of these cell lines express high levels of EGFRs, whereas the BT-20 cell line has a high EGFR level (233,000 receptors/cell) but has low GCF mRNA level.

The GCF cDNA contains homology to a cDNA that encodes a 4.5 kb mRNA. A cDNA that encodes eighty percent of this mRNA has been isolated and sequenced. This cDNA contains an open reading frame of 2112 nucleotides which would yield a protein of 78 kDa. This protein would have a pI of 4.3 and would be rich in glutamic acid residues. It would also contain a basic region similar to the DNA binding region of GCF. Fluorescence in situ hybridization was used to localize the gene for this cDNA to chromosome 20q13.3. This locus has been shown to be amplified in some breast cancer cell lines.

To investigate the gene structure of GCF and the relationship with the cDNA encoding the 4.5 kb mRNA, we have isolated P1 genomic clones containing the gene. Analysis of 5' region of the gene should clear up the questions pertaining to the initiation of translation and homology to other RNA species.

Many of the transcription factors identified for EGFR gene expression have been shown to bind downstream of the major in vivo transcription initiation site. We have identified a second transcriptional repressor for the EGFR gene. EGFR transcriptional repressor (ETR) was shown to bind to a TTCGAGGG sequence located 600 base pairs upstream of the major *in vivo* transcriptional start site. Deletion analysis of the region -877 to -870 leads to increased EGFR promoter activity. Fusion of this region to the thymidine kinase promoter driving chloramphenicol acetyltransferase results in lower CAT activity. Binding and crosslinking experiments identify a 128 kDa protein found in A431 and nuclear extracts as the protein that has the repressor activity.

Publications:

Hou, X, Johnson AC, Rosner MR. Identification of an epidermal growth factor receptor transcriptional repressor, J Biol Chem 1994;269:4307-12.

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

PROJECT NUMBER
Z01 CB 08010-21 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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	NCI
Others:	K. Chang	Visiting Associate	LMB, NCI
	A. Rutherford	Biologist	LMB, NCI

COOPERATING UNITS (If any)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Ultrastructural Cytochemist Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

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

B

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

To deliver toxins or other therapeutic agents to cancer cells, it is necessary to have antibodies that selectively recognize these cells. One approach to this problem is to identify differentiation antigens that are present on normal nonessential tissues and continue to be expressed on cancers derived from them. We have isolated two monoclonal antibodies that have these properties. One isolated by K. Chang is MAb K1. It reacts with a differentiation antigen present on the surface of normal ovary and on most ovarian cancers. The antigen is a 40 kDa glycosylated protein linked to the cell surface by phosphotidyl inositol. To isolate the antigen, K. Chang has screened expression libraries made from ovarian cancers and ovarian cancer cell lines. Initially, a cDNA encoding a 34 kDa protein was isolated and sequenced. This is a cytosolic protein that is abundant in ovarian cancer cells but is not the CAK1 antigen. Therefore, additional screening was carried out and recently a partial cDNA clone was isolated which has properties expected for the antigen present in ovarian cancer cells. Further sequencing and epitope mapping are currently underway to establish its identity.

The other monoclonal antibody is PR1, an IgM that reacts with the surface of normal prostate, almost all primary prostate carcinomas, and many metastatic prostate cancers. It is expressed in very few other normal tissues or non-prostate tumors. K. Chang has identified a protein which reacts with the PR1 antibody. It is a 34 kDa protein found in the membrane fraction of LNCaP cells and it is induced by growth of these cells on laminin. By screening an expression library made from human prostate, several clones were obtained that react with the PR1 antibody. One of these has the sequence of the laminin binding protein isolated by M. Sobel (NCI) and coworkers. Other clones with different sequences have also been isolated. The relationship of these cDNA clones to the PR1 antigen is under investigation.

Publications:

Chang K, Pastan I. Molecular cloning and expression of a cDNA encoding a protein detected by the K1 antibody from an ovarian carcinoma (OVCAR-3) cell line, Int J Cancer 1994;57:90-7.

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

PROJECT NUMBER
Z01 CB 08710-17 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

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)*
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LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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

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

We have been studying the mechanism of action of molecular chaperones and their role in DNA replication. We found that three *E. coli* heat shock proteins, DnaJ, DnaK (the Hsp70 homologue), and GrpE are required for plasmid P1 DNA replication *in vitro*. We discovered 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 with high affinity to *ori*P1 DNA. GrpE 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 μM by a metal ion buffer system. GrpE specifically lowers the concentration of Mg+2 required for ATP hydrolysis by DnaK, suggesting that this may be the step in RepA activation where Mg+2 is essential. We have tested other molecular chaperones in the RepA activation system. We discovered that ClpA, the regulatory component of the ATP-dependent ClpAP protease, activates the sequence specific DNA binding activity of RepA. ClpA forms an ATP-dependent complex with RepA and converts RepA dimers to monomers with ATP hydrolysis. ClpA also acts as a chaperone in protecting luciferase from irreversible heat inactivation but unlike DnaJ, DnaK and GrpE it is unable to reactivate the inactivated luciferase. These results demonstrate that ClpA is a new molecular chaperone.

Major Findings:

Molecular chaperones participate in protein folding pathways by recognizing and modulating partially folded proteins during protein synthesis, oligomeric structure formation, complex and aggregate disassembly and protein degradation. The *E. coli* DnaJ, DnaK and GrpE chaperones play a critical role in several *in vitro* systems, including in *ori*P1 and *ori*λ DNA replication and the refolding of denatured luciferase, rhodanese and RNA polymerase.

For *ori*P1 DNA replication DnaJ, DnaK and GrpE mediate the activation of the sequence-specific DNA binding activity of RepA, the replication initiator protein. RepA dimers are inactive for DNA binding but form a tetrameric complex with DnaJ. The RepA-DnaJ complex targets RepA for recognition by DnaK. DnaK then dissociates the RepA dimers into monomers in an ATP-dependent reaction. RepA monomers bind with high affinity to *ori*P1 DNA without further involvement of DnaJ and DnaK.

Initially the RepA activation reaction *in vitro* with DnaJ and DnaK was puzzling because, unlike the *in vivo* situation, GrpE was not required. However, we have found conditions that mimic the physiological situation. GrpE is absolutely necessary for RepA monomerization *in vitro* when the free Mg^{+2} 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^{+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 DnaJ and DnaK. Our results imply that GrpE facilitates the utilization of Mg^{+2} for an essential step in RepA activation.

Since GrpE lowers the concentration of Mg^{+2} required for RepA activation, we wanted to know if it affects the concentration of Mg^{+2} required for ATP hydrolysis by DnaK. We have found that GrpE specifically lowers the concentration of Mg^{+2} required for ATP hydrolysis by DnaK by about four fold. Using the same conditions, DnaJ or DnaJ and RepA did not affect the Mg^{+2} requirement for ATP hydrolysis, however they stimulated the rate of hydrolysis. ATP binding by DnaK was unaffected by limiting Mg^{+2}. These results suggest that ATP hydrolysis may be the step in RepA activation where Mg^{+2} is essential.

We have used the RepA activation system as a tool to identify new molecular chaperones and to learn about the specificity of the system. We discovered that ClpA, in an ATP-dependent reaction, performs the same function as DnaJ and DnaK in activating the sequence specific DNA binding activity of RepA. ClpA is the regulatory component of the ATP-dependent ClpAP protease of *E. coli*. It has properties that are reminiscent of those of molecular chaperones in that it is a member of a ubiquitous family of highly conserved proteins present in multiple forms in all organisms tested, including bacteria (ClpA, ClpB, ClpX, ClpY), yeast (Hsp104, Hsp78), plants (ClpC), hamsters and humans.

To understand the mechanism of activation by ClpA, we determined whether RepA activated by ClpA could be isolated free from ClpA. We demonstrated by gel filtration chromatography that activated RepA is separable from ClpA and remains in its activated

form. We looked for a ClpA-RepA protein complex to probe the mechanism of activation by ClpA in more detail. When we simply chromatographed a mixture of RepA and ClpA without ATP on a gel filtration column, ClpA and inactive RepA were separated from each other. However, when we incubated RepA and ClpA with ATP-γS before gel filtration chromatography, ClpA and RepA eluted together in the excluded volume.

Our results show that RepA forms a complex with ClpA in the presence of nucleotide and can be chased to its active form upon ATP hydrolysis.

We analyzed ClpA-activated RepA to determine if ClpA, like DnaJ and DnaK, dissociates RepA dimers into monomers. Activated RepA eluted on gel filtration as a 33 kDa protein. RepA that had not been activated by ClpA eluted as a 68 kDa protein, the dimer of 32 kDa monomers. Furthermore, the electrophoretic mobility on SDS-PAGE of RepA activated by ClpA was indistinguishable from that of 32 kDa RepA subunits. Therefore ClpA activates RepA by converting RepA dimers to monomers.

To determine if ClpA could function as a molecular chaperone in another system, we tested it in the luciferase reactivation system in which DnaJ, DnaK and GrpE protect luciferase from irreversible heat inactivation and reactivate it. ClpA was unable to reactivate luciferase. However, ClpA present during the heat treatment with luciferase prevented irreversible inactivation of luciferase such that DnaJ, DnaK and GrpE could reactivate luciferase. This result suggests that ClpA prevents aggregation of luciferase and shows that there is specificity in the interaction of a chaperone with its target proteins.

Since ClpA activates the ClpP peptidase for ATP-dependent proteolysis, we tested whether RepA was a substrate for ClpAP protease. We found that ClpAP protease degraded RepA. ClpA was required for degradation, not simply for monomerization, since RepA activated by DnaJ and DnaK was not degraded by ClpP alone but was degraded by ClpAP. Furthermore, RepA bound to *ori*P1 DNA was protected from degradation by ClpAP.

Our working model for ClpA function in activation or degradation of RepA is analogous to our model for DnaJ, DnaK and GrpE function. In the RepA activation reaction ClpA binds to RepA dimers in the presence of ATP, performing the targeting role of DnaJ. Very likely, ClpA changes conformation upon ATP hydrolysis, and this results in the release of RepA monomers. Thus, ClpA also performs the ATP-dependent protein remodeling function of DnaK. The released RepA monomers bind to *ori*P1 DNA. ClpA also targets RepA for ClpAP degradation. Perhaps this pathway is analogous to the function of the heat shock proteins in the degradation of abnormal and heat aggregated proteins.

Publications:

Skowyra D and Wickner S. The Interplay of the GrpE Heat Shock Protein and Mg^{+2} in RepA Monomerization by DnaJ and DnaK, J Biol Chem 1993;268:25296-25301.

Wickner S, Skowyra D, McKenney K. The function of Heat Shock Proteins in Phage and Plasmid DNA Replication, Semin in Virol 1994;in press.

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

PROJECT NUMBER
Z01 CB 08714-17 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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
	H. Munavar	Special Volunteer	LMB, NCI
	D. Sledjeski	IRTA Fellow	LMB, NCI
	Y. Jubete	Visiting Fellow	LMB, NCI
	W.-f Wu	Visiting Fellow	LMB, NCI

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Biochemical Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.0	6.0	1.0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

We have continued studies on the role that protein degradation plays in regulating gene expression and on the linkages between chromosome replication and partition of chromosomes during cell division. Studies have begun on the recognition signals within targets of the Lon energy-dependent protease. Turnover of the lambda N protein by the Lon ATP-dependent protease requires signals throughout the substrate, since full-length GST-N fusions are normally degraded by Lon while similar fusions lacking portions of either the N-terminus or C-terminus are either stable or degraded in a Lon- independent fashion. *E. coli* contains an alternative energy-dependent protease, Alp, whose substrate specificity overlaps with that of Lon but is expressed only under special conditions. We have now isolated strains which have this protease expressed at high levels and are analyzing candidate mutations in the protease. We have continued to examine the regulation of synthesis of RcsA, the unstable positive regulator of capsule synthesis which is also a substrate for Lon and Alp. *rcsA* is silenced by HNS, a histone-like protein. DsrA, a small RNA which positively regulates RcsA synthesis, has been found to have global effects on *E. coli*, turning up other HNS-silenced operons and decreasing the cellular amounts of HNS. DsrA may carry out a novel role as an RNA regulator of transcription. The second general class of ATP-dependent proteases in *E. coli* is exemplified by ClpAP and ClpXP, two-component proteases in which the substrate specificity is conferred by the ATPase subunits (ClpA or ClpX). Mutations in the protease subunit, ClpP, demonstrate the separation of activities specific for ClpA-dependent and ClpX-dependent substrates, as well as processing of ClpP itself. Work on cell cycle mutants has focussed on *mbrA4*, which appears to be altered in the control of initiation of DNA replication. New, more stringent alleles of *mbrA* are being isolated by localized mutagenesis, and the basis for lethality of *mbrA4* in hosts devoid of RNAseH is being investigated using a conditional allele of *rnhA*. Cloning *mbrA4* has been complicated by the inability to detect phenotypes for the gene when present on the plasmid, either because it is silent or inactivated. By using flanking, selectable markers, we are now able to detect movement of the *mbr*A region between plasmid and chromosome, which should allow cloning and genetic manipulation even in the absence of a direct *mbrA* plasmid phenotype.

Major Findings:

1. Lon:

We have found that the heat shock chaperones DnaJ and DnaK are necessary for the degradation of abnormal proteins by the ATP-dependent protease Lon, while the naturally unstable Lon substrates SulA and N are degraded in a manner which is independent of DnaJ and DnaK. RcsA, another naturally unstable Lon substrate, requires DnaJ and DnaK to remain in an active conformation under many growth conditions, and for rapid degradation of RcsA. Analysis of the state of RcsA in *dnaJ* mutant cells suggests that the inactivity and decreased degradation of RcsA reflect the presence of RcsA in inaccessible aggregates. These observations suggest that the requirement for DnaJ and DnaK for degradation of abnormal proteins may also be to allow misfolded proteins to remain soluble, in a form accessible to proteases.

To investigate the recognition elements within Lon substrates, we have examined the in vivo degradation of derivatives of lambda N protein fused to GST, obtained from N. Franklin. A full length GST-N fusion is degraded in *lon*$^+$cells and degradation is slowed in *lon* mutants; as for the wild-type N protein, degradation is independent of DnaJ. Degradation continues through the GST moiety. However, all tested GST-N fusions carrying fragments of N are no longer degraded in a Lon-dependent manner, suggesting that the recognition elements include much of the N protein. Shorter deletions and hybrid proteins of N and a related, stable protein, Nun, will be used to identify essential elements.

2. Alp:

The Alp protease is defined as an activity capable of substituting for the Lon protease to degrade SulA and RcsA. We have previously shown that the protease is active in cells which are mutant for SsrA, a small stable RNA, and which contain a kanamycin resistance gene present on a plasmid. As a first step to isolating mutations in the protease itself, we identified a mutation which increases protease activity in cells without the kanamycin resistance gene. Isolation of this mutation has simplified the search for mutations in the protease itself; several promising candidates for mutations in the protease are being analyzed. In addition to providing a useful comparison for Lon in terms of substrate selection, identification of the coding genes for the Alp protease will allow an understanding of the role of SsrA and the kanamycin resistance phosphotransferase in modulating protease activity or expression.

3. Capsule Synthesis: Regulation of RcsA expression:

Because RcsA is a positive regulator of transcription which is limiting for synthesis of capsular polysaccharide, we have been interested in examining the regulation of *rcsA* synthesis. *rcsA-lacZ* fusions which contain at least 97 bp upstream of the start point of transcription are expressed at low levels; fusions with only 59 bp upstream are expressed at very high levels. The longer fusions are negatively regulated, or "silenced", by interaction with HNS, a histone-like protein implicated in the negative regulation of multiple operons in *E. coli*. Overproduction of a small (85 nucleotide) RNA, DsrA, increases *rcsA* expression, apparently by overcoming the HNS silencing. DsrA over-production also turns up expression of other HNS silenced operons, and decreases the level of HNS in the cell. Analysis of mutations in DsrA, however, demonstrates that the decreased level of HNS is not responsible for the increase in *rcsA* expression; a mutant RNA that shows higher levels of stimulation of *rcsA* has very little effect on HNS levels. There is no simple parallel between the effect of various mutants in DsrA on different HNS-silenced operons. The precise mechanism of action of

the small RNA is still unclear, but it may interact directly with DNA in the region of the operon with which HNS interacts. We believe the RNA is part of a regulatory mechanism used under as yet unknown conditions of stress to rapidly increase capsule synthesis by increasing RcsA synthesis.

4. Clp:

The Clp family of energy-dependent proteases includes ClpAP, containing the ClpA ATPase subunit and ClpP, a protease subunit, and ClpXP, in which ClpX serves as the ATPase subunit, again with ClpP. *In vivo*, these two complexes have different substrate specificities.

In collaboration with Sue Wickner, we have been investigating the possible in vivo role of ClpA as a molecular chaperone. She has demonstrated such a role *in vitro* for ClpA with respect to activation of the RepA protein; previously DnaJ, DnaK and GrpE had been shown to act as a chaperone for RepA activation *in vivo* and *in vitro*. *In vivo*, mutations in *clpA* or *clpP* do not change the apparent *in vivo* activity of RepA for replication or transcriptional regulation. Overproduction of ClpA in a *dnaJ* mutant host leads to the inability of cells to grow at temperatures of 32°C or above; this extreme temperature sensitivity may reflect an incomplete chaperone-like activity of ClpA *in vivo*. *In vivo* studies of site-directed mutations in ClpA made by S.K. Singh in Michael Maurizi's laboratory demonstrate that protease activity is not necessary for this temperature sensitivity in *dnaJ* hosts, and also demonstrate that one mutant form of ClpA acquires a new activity, demonstrated by poor growth in a *clpP*$^+$ but not a *clpP* mutant host. Thus this mutation may identify part of a site involved in substrate recognition/selection.

Using a set of *in vivo* assays of Clp activities, we have screened plasmids containing mutagenized *clpP*, as well as some site-directed mutations in conserved residues of *clpP* for ability to degrade either ClpAP or ClpXP substrates. The initial characterization of these mutants suggests that recognition of ClpA and ClpX can be separated, as can activity for processing of ClpP itself. Further isolation of mutants and *in vitro* analysis of these proteins in collaboration with Dr. Michael Maurizi should provide an excellent functional analysis of ClpP.

ClpY was identified in the sequence data base as a close relative of the ATPase subunit ClpX; others have recently shown that it is part of a heat shock operon in *E. coli*. The first gene in this operon has homology to the proteasome subunits of eukaryotic ATP-dependent proteases, making it likely this operon encodes yet another energy-dependent protease. We have cloned the *E. coli* operon and are constructing mutants in the genes to examine the *in vivo* role of these proteins, including their ability to substitute for Clp.

5. Cell cycle mutations:

We have been studying the *E. coli* cell cycle using a series of diploid mutants that we isolated as resistant to camphor vapors (*mbr* mutants). The original eleven mutants define four genetic loci. Initial characterization of the mutants indicated that one of them, *mbrA4*, is altered in the control of initiation of DNA replication. We have been focusing on the *mbrA* locus.

The molecular analysis of *mbrA* has been hindered by the inability to clone the gene. Either *mbrA4* is lost when present even on low copy number plasmids, or it is phenotypically silent on the plasmid. We have now identified plasmids which carry markers flanking *mbrA*; we can select for these markers and then monitor for the presence of *mbrA* after returning the region to the chromosome.

One phenotype of *mbrA4* identified in the initial characterization was the inability of *mbrA4* to be maintained in a strain where initiation of DNA replication is uncoupled from the cell cycle (*rnhA*⁻ mutant). We have built a strain containing *mbrA4* and a conditional *rnhA* gene to allow us to determine the cause of this lethality and select for mutants that allow *mbrA4* to be maintained in the absence of controlled DNA initiation. This should further define components that interact with *mbrA* and participate in initiation of DNA replication.

To understand the role of *mbrA* in the cell cycle, we would like to define other cellular components that interact with it, using second site suppressor analysis. This would be simplified by the isolation of *mbrA* mutants with tighter phenotypes than *mbrA*4. Localized mutagenesis has yielded 100 new potential *mbrA* mutants. Many of the mutants appear to have more severe defects than *mbrA4*; appropriate mutants will be used to isolate and analyze second site suppressors.

Publications:

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Painbeni E, Mouray E, Gottesman S, Rouviere-Yaniv J. An imbalance of HU synthesis induces mucoidy in *Escherichia coli*. J Mol Biol 1993;234:1021-37.

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

PROJECT NUMBER
Z01 CB 08750-14 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Genetic Regulatory Mechanisms in *Escherichia coli* and Its Bacteriophage

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

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Developmental Genetics Section

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	4.0	0.5

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

B

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

We are studying how the cyclic AMP receptor protein (CRP) activates transcription in *Escherichia coli.*

(i) We have found that the structural integrity of the DNA is important in CRP activation of *lac* transcription. We have shown that for activation to occur, DNA must be intact with proper Watson-Crick base pairing. Changing the DNA sequence between where CRP is bound and the *lac* promoter can also affect CRP-mediated transcription activation. We have proposed that there is transmission of some signal from CRP through the DNA to the promoter.

(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 *lac* promoter 25-fold compared to 5-fold stimulation seen in the presence of cAMP. We have proposed that adenylate cyclase interacts with CRP during the time cAMP is translocated to CRP.

(iii) The *pts* operon encodes protein necessary for uptake of many sugars and for activation of adenylate cyclase. We 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. *pts* is also regulated by glucose and by the fructose repressor. *pts* is subject to post-transcriptional regulation since RNA from one promoter is subject to RNase E degradation.

(iv) DNA with as few as four mismatched bases can serve as a promoter when placed near the *lac* promoter. Transcription from the "mismatch promoter" has all the characteristics of a *bona fide E. coli* promoter.

Major Findings:

The cyclic AMP (cAMP) receptor protein (CRP) of *Escherichia coli* is a DNA binding protein that regulates transcription of many operons. To activate transcription, CRP, liganded to cAMP, binds to sites upstream from promoters and causes transcription to initiate more readily. We are studying the precise mechanism by which CRP activates transcription with the aim of determining the molecular interactions and any other factors involved in activation.

I. Transcription activation by CRP

A. The role of DNA structure in transcription activation: It is known that CRP can interact with RNA polymerase and that this interaction is necessary for transcription activation. We are trying to determine whether this protein-protein interaction is sufficient for activation. We found that disruptions in the DNA double helix in the spacer region between the CRP binding site and the *lac* promoter prevented CRP activation of transcription. Disruptions that prevented activation were single-stranded gaps of two or more bases; nicks (single-strand breaks) or 1-base gaps had little effect. We showed that CRP and RNA polymerase were still interacting normally, even when the DNA contained 4-base single-stranded gaps, by quantitative DNase footprinting. Our conclusion is that CRP-RNA polymerase interaction is necessary but not sufficient for CRP activation of transcription.

We are investigating the basis for the requirement for structural integrity of the DNA between CRP and RNA polymerase. Since gapped DNA prevented CRP activation of transcription, we tested DNA that had both strands intact, yet mismatched. We found that, like the experiments with the gapped DNA, DNA that had two or more bases mismatched was severely defective in transcription activation. We conclude from these experiments that it is necessary to have proper base-pairing in this spacer region in order to have CRP activation of transcription.

We have proposed and tested the model that CRP binding to DNA passes a signal from the CRP binding site to the promoter to enhance transcription initiation. A possibility that is consistent with our requirement for intact, base-paired DNA would be that CRP shifts the DNA into an A or A-like form. Since DNA is in the A form at the moment of transcription, having it already in the A form would enhance initiation. We are investigating whether a particular sequence within the spacer region is necessary for CRP to activate transcription. We changed the sequence in the spacer region to one that should go more readily into the A form (CCCCGGGG) and compared it to the wild type and to one that would be not A form-philic. We found that the A form-philic DNA had about 25% more activation of transcription by CRP than did the wild type. We are currently using site-directed mutagenesis to mutagenize the spacer region. Preliminary results suggest that almost any sequence is tolerated in this region, i.e., CRP can activate transcription very well regardless of the sequence of the region.

B. Interaction of CRP with other factors:

1. We have investigated whether there are other factors involved in CRP activation of transcription. We had previously found from fluorescence experiments that there is an interaction between CRP and adenylate cyclase, although the experiments were limited by inability to have adenylate cyclase in high enough concentration without it aggregating. We have found that adenylate cyclase enhances CRP activation of transcription when adenylate cyclase and ATP are used to replace cAMP in an *in vitro* transcription assay. This stimulation is likely to be through enhanced binding of CRP to DNA, because we found that adenylate cyclase stimulates CRP binding in a gel mobility shift assay.

We have proposed that the interaction of CRP and adenylate cyclase places the CRP in a conformation that can bind readily to DNA.

2. In a search for additional factors that can affect CRP activity, we have isolated *E. coli* mutants that are phenotypically CRP^- yet are genotypically crp^+. These mutations map to at least four different loci on the *E. coli* chromosome and are being more finely mapped and characterized.

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

The *ptsHI* operon of *E. coli* encodes the proteins necessary for the uptake of glucose and other sugars, HPr and Enzyme I, and a glucose specific protein, Enzyme III. We are interested in the *pts* operon for two reasons: it is regulated transcriptionally by CRP and cAMP, and its gene products (Enzyme I and Enzyme III) have been implicated in regulation of adenylate cyclase activity.

1. Transcriptional regulation of *pts*: We had previously demonstrated that the *pts* operon is controlled by two promoters, each of which is regulated by CRP and cAMP. Furthermore, each of these promoters can switch transcription initiation points, depending on the presence or absence of CRP and the degree of supercoiling. Which promoter is used can have consequences on how much gene product is made (see below).

Glucose is known to regulate the activity of adenylate cyclase in a negative fashion. Since cAMP aids *pts* expression, then glucose would have a negative effect on pts expression. However, glucose increases the expression of *pts in vivo*, suggesting another mechanism for glucose regulation. Recently, the fructose repressor FruR was shown to have a binding site in the vicinity of the *pts* promoters. The effect of FruR on transcriptional regulation of *pts* expression was studied to test whether FruR is a glucose-inducible repressor responsible for the new glucose effect. We found that FruR does repress one of the promoters of *pts* but not the one that is "induced" by glucose addition. This suggests that the mediator of the glucose stimulation of *pts* expression seen *in vivo* is not FruR and is currently being investigated.

2. Post-transcriptional regulation of *pts*: The RNAs produced from each promoter and from each start site have different potential secondary structures. Such secondary structure could affect the stability of each mRNA differently, affecting final expression of the operon. By analyzing the sequences of the different RNAs produced, we hypothesized that RNA from one of the promoters could be a substrate for RNase E, an enzyme that degrades mRNA at the 5' end. We found that that particular RNA is indeed sensitive to RNase E degradation, *in vitro* and *in vivo*. This may mean that the relative amounts of the gene products from the multicistronic *pts* operon would differ depending upon which promoter is used.

III. An artificial promoter

In our experiments to test whether mismatched DNA has an effect on CRP activation of lac transcription, we found that a 4-base mismatch could serve as a functional RNA polymerase binding site or promoter. Transcription initiates at the first template T residue that is downstream of the mismatched sequence. This mismatched DNA promoter acts like a *bona fide E. coli* promoter in that it is sigma-dependent and inhibited by rifampicin. Transcription from the mismatched DNA may require another promoter close by, since deletion of the nearby lac promoter prevented transcription from the mismatched DNA promoter. Mismatched DNA serving as a promoter may have implications in DNA repair mechanisms and in DNA replication initiation.

Publications:

Barber A, Zhurkin V, Adhya S. CRP-binding sites: evidence for two structural classes with 6-bp and 8-bp spacers, Gene 1993;130:1-8.

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

PROJECT NUMBER
Z01 CB 08751-14 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Regulation of the *gal* regulon of *Escherichia coli*

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

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Developmental Genetics Section

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0.0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

We are studying the mechanism of control of expression of genes of D-galactose transport and metabolism in *Escherichia coli*. We have previously demonstrated that the *gal* regulon contains at least five operons, two of which, *galR* and *galS*, encode for two repressors. We have shown that GalR and GalS repressors which are homologous negatively regulate the *gal* regulon members independent of each other and without hetermer formation. GalR and GalS are inactivated by galactose for induction of the *gal* operon and other members of the regulon. Using a *gal* operon DNA construction in which the two *gal* operators have been replaced by *lac* operators, we have shown that DNA loop formation in the promoter segment via interaction of *O*E and *O*I bound LacI repressor is essential for normal repression of the two constituent promoters, *galP1* and *galP2*, in a purified system. With wild type *gal* DNA in the same system, GalR and GalS are unable to form loop and repress the promoters. Under these conditions, only *galP1* is partially repressed and *galP2* not at all. This suggests that in a purified system GalR and GalS need a looping factor, presumably a protein. We have isolated *E. coli* mutants presumed to be defective in the looping factor. These mutants are currently being characterized.

DNA loop formation have been assayed by measuring the appearance of DNase hyper-sensitive sites in the looped region. We have found that under such conditions, RNA polymerase can still bind to the *gal* promoter, showing that repressor inhibits transcription at a post-RNA polymerase binding step. We are investigating the model that repressor inhibits RNA polymerase activity by a direct protein-protein contact which is facilitated by DNA looping.

In studying the DNA-GalR-ligand interactions, we have identified circular dichroism spectral changes, which are characteristic of GalR interactions with DNA and galactose. We have also identified two amino acid residues in GalR which are involved in galactose binding.

We have discovered a new cistron, *galM*, in the *gal* operon that encodes a mutarotase for galactose. The mutarotase converts the β-galactose moiety of lactose to α-galactose *in vivo* for futher metabolism.

Major findings:

The genes of D-galactose transport and metabolism are negatively regulated by Gal repressor (GalR) and Gal isorepressor (GalS) and constitute a regulon. The following results and conclusions highlight our research during the year 1993-1994.

1. Mechanism of repression of the gal operon by GalR and LacI. The repression of the two promoters, *galP1* and *galP2*, of the *gal* operon requires binding of GalR to two operators, O_E and O_I, which encompass the two promoters and are separated by 11 B-DNA helical turns. The two DNA-bound repressors interact forming a DNA loop. Replacement of the two *gal* operators by *lac* operators, O_E^L and O_I^L, brings about repression of the *gal* operon under Lac repressor (LacI) control. LacI efficiently represses both *galP1* and *galP2* in a O_E^L-O_I^L DNA template in a purified transcription system *in vitro*. Complete repression of *galP1* and *galP2* by LacI requires DNA looping. Sliding the position of the promoters with respect to O_E and O_I and enlargement of the loop size have shown that repression by LacI occurs irrespective of the promoter location and as long as the distance between the two operators permit loop formation. As shown before, GalR is unable to form DNA loop *in vitro*, and an additional factor is needed for repression by GalR on O_E^G-O_I^G DNA in the *in vitro* transcription system. We have isolated 24 *E. coli* mutants defective in the putative looping factor using a genetic selection system which consists of two tandemly arranged *galP2* promoter--one fused to *lacZ* gene and the other to *cat* gene--and a multicopy *galR* gene. In this system, we selected for mutants with Lac^+Cat^R phenotype in the absence of inducer. Interestingly, some of these mutants are conditional lethal for growth. They are currently being analyzed.

2. Collective binding of RNA polymerase and repressor in gal operon. By DNase protection experiments, we have shown that LacI mediated DNA looping in the *gal* operon generates periodically arranged DNase hypersensitive sites in the looped segment. In this state, RNA polymerase is still able to bind to the promoters. Such collective binding of RNA polymerase and repressor has also been demonstrated by gel electrophoresis assay of DNA-protein complexes. The failure to hinder RNA polymerase binding by repressor suggests that repressor inhibits transcription by a different mechanism. We are currently studying the model that repressor acts by inhibiting RNA polymerase activity by a direct protein-protein contact which is facilitated by DNA looping.

3. Characterization of GalS. The GalS isorepressor has been purified to homogeneity by a combination of ion exchange, hydroxyapatite, and low salt precipitation steps. Gel filtration experiments indicate that GalS, like GalR, is a dimer at 1 to 50 μM concentrations. Antibody raised against purified GalR and GalS show that the two proteins cross-react with their antibodies, consistent with the 85% similarity of their amino acid sequences. *In vitro* DNA binding and transcription studies with *gal* and another member of the regulon, *the mgl operon*, has shown that the two repressors act by binding to the same operator sites but do not regulate *gal* and *mgl* in a parallel fashion. GalR is more effective in binding to and repressing *galP1*; whereas, GalS binds and represses *mgl* promoter better. Both GalR and GalS repress only *galP1* but not *galP2* in a purified system. Repression of *galP1* requires binding to only the O_E operator and presumably through a contact with the promoter-bound RNA polymerase. We suggest that GalS, as GalR, requires a looping factor for complete repression of *galP1* and *galP2* by DNA looping *in vitro*. Both GalR and GalS are sensitive to D-Galactose (inducer) in specific DNA binding and in transcription repression *in vitro*. GalR and GalS do not form hybrid repressors.

4. Ligand binding of GalR. We have continued our studies on the characterization of binding of D-galactose to GalR protein. We have previously identified two sugar binding amino acids residues by mutational analysis: S184F and Y244F. These two sites were predicted to be in the sugar binding domain. The two mutant proteins were insensitive to galactose both in GalR-DNA complex formation and in *in vitro* GalR mediated transcription repression of *galP1*. Circular dichroism spectral analyses of GalR and its complexes with DNA and sugar have revealed characteristic spectral shifts upon complex formation. Such characteristics are now being tested with the sugar-nonbinding GalR mutant proteins.

5. Mutarotation of galactose is catalyzed in vivo by an enzyme encoded in the gal operon. We have identified an enzyme, mutarotase, which is encoded by a newly discovered cistron, called *galM*. We have found that *galM* is part of the *gal* operon of *E. coli*. Although mutarotation occurs spontaneously in water, we have been able to demonstrate genetically that such a rotation is largely enzyme catalyzed *in vivo*. The β-galactose anomer generated intracellularly by hydrolysis of lactose by β-galactosidase must be converted to α-galactose for subsequent phosphorylation by galactokinase. We have shown that a strain of *E. coli* which has been engineered to have the *galM* gene removed is largely defective in the utilization of the galactose moiety of lactose.

Publications:

Zwieb C, Adhya S. Plasmid vectors for the analysis of DNA bending. In: Kneale GG, ed. Methods in Molecular Biology. Totowa: Humana Press Inc., 1994;281-294.

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

PROJECT NUMBER
Z01 CB 08752-14 LMB

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October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mechanisms of Thyroid Hormone Action in Animal Cells

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

I. Structure and activity of the human β1 thyroid hormone receptor (h-TRβ1).

A. The nature of thyroid hormone binding site of h-TRβ1: Based on the binding data of five thyroid hormone analogs to eight naturally occurring mutated h-TRβ1 and the structure of the hormone binding domain as an eight-stranded α/β barrel, we proposed the location of the hormone, 3,3',5-triiodo-L-thyronine (T3), in the binding site of h-TRβ1. Ionic bonds anchor the hormone's alanine side chain to loop 4 of the 8-fold α/β barrel. The phenyl ring lies across the amino-terminal face of the domain with the phenoxy ring pointing downward into the barrel interacting with β-strand 8 on the opposite side. Loops 1 and 7, which are located on the same face as the DNA binding domain, fold over the top of the barrel toward the bound hormone. The present model should provide a basis for further studies to understand the T3-dependent transcriptional activity of h-TBβ1.

B. Binding of T3 induces conformational changes in h-TRβ1: We asked the question as to whether T3-dependent transcriptional activity of h-TRβ1 is mediated by conformational changes induced by T3. Confomational changes were found in the regions at or near Lys201, Arg238, Lys239 and Trp234. These residues are located on the amino terminal face of the barrel which is on the same side of the DNA binding domain. The conformational changes could conceivably act as a signal to affect the transcriptional activity of h-TRβ1.

II. The molecular basis of generalized resistance to thyroid hormone (GTRH). To understand the molecular basis of differential phenotypic expressions in kindreds with the same genotypes, we studied the effects of tissue-specific factors on the dominant negative potencies of GRTH mutants. Transactivation activity was compared in different cell types using several naturally occuring thyroid hormone responsive elements (TREs). The dominant negative potency is TRE- and cell type-dependent, indicating the important role of tissue-specific factors in mediating the phenotypic expression of mutant receptors in GRTH patients.

III. Thyroid hormone and tumorigenesis. The role of TR in hepatocarcinogenesis was studied by characterizing the TRs in nine human hepatocarcinoma cell lines. The degree of differentiation was found to be inversely correlated to the level of TRβ1 protein. Overexpressed h-TRβ1 was found to mediate the proliferation of poorly differentiated human hepatoma cells.

Major Findings:

I: Structure and activity of the human β1 thyroid hormone receptor (h-TRβ1).

A. The nature of thyroid hormone binding site of h-TRβ1.

Thyroid hormone nuclear receptors (TRs) are members of the steroid hormone/retinoic acid receptor superfamily. They are transcriptional factors which regulate growth, differentiation, and development. The TR-mediated activation or repression of target genes is dependent on the thyroid hormone, 3,3',5-triiodo-L-thyronine (T_3). At the present time, the molecular basis for the T_3 dependence is not clearly understood. As a first step to understand how the hormone signal is transduced from the hormone binding domain to the DNA binding domain to affect the gene regulatory activity of h-TRβ1, we studied the nature of the thyroid hormone binding site.

We utilized the naturally occurring mutant receptors which were derived from patients with the syndrome of generalized thyroid hormone resistance as a tool to probe the topography of the T_3 binding site. Each mutant has a point mutation in the hormone binding domain (KT, R338W; TP, L450H; IR, D322H; NN, G347E; AH, P453H; OK, M442V; RL, 459C; and ED, A317T). Compared to the wild-type h-TRβ1, binding of T_3 was reduced by as much as 97% for the mutants. The order of binding affinity of wild-type h-TRβ1 to the analogues is T_3>D-T_3>L-thyroxine>3,5-diiodo-L-thyronine>3,3',5'-triiodo-L-thyronine. The mutant receptors showed essentially the same order of reduced affinities for the analogues, but the amounts of the reductions varied in each case. These results suggest specific local interactions (interplay) of analogues with the mutated residues in the receptors. On the basis of these data and a putative structure of the hormone binding domain as an 8-stranded α/β barrel, we proposed the location of the hormone in the binding site of h-TRβ1. Ionic bonds anchor the hormone's alanine side chain to loop 4 of the 8-fold α/β barrel. The phenyl ring lies across the amino-terminal face of the domain with the phenoxy ring pointing downward into the barrel interacting with β-strand 8 on the opposite side. Loops 1 and 7, which are located on the same face as the DNA binding domain, fold over the top of the barrel toward the bound hormone. The present model should provide a basis for further studies to understand the T3-dependent transcriptional activity of h-TRβ1.

B. Binding of T3 induces conformational changes of h-TRβ1.

By analogy with the steroid hormone receptors, four domains could be assigned to h-TRβ1, A/B (Met^{1}-Leu^{101}), C (Cys^{102}-Met^{169}), D (Ala^{170}-Lys^{237}) and E (Arg^{238}-Asp^{456}). Domain E was previously thought to be the T_3 binding domain. However ,we have recently shown that domain E alone does not bind T_3 and requires domain D to become a functional T_3 binder. Using circular dichroism and sequence analyses, we have recently proposed that TRβ1 is essentially composed of two functional domains; the DNA binding domain of known structure, linked by an α-helix (Ala^{170}-Gln^{200}) to a hormone binding domain (Lys^{201}-Asp^{456}) with an α/β barrel structure. It is possible that the hormonal signal for activation or repression of T_3 target genes is transmitted from the hormone binding domain via the α-helical hinge (Ala^{170}-Gln^{200}). This would mean that T_3 induces structure changes either in the hormone binding domain or the helical hinge or both.

To understand the molecular basis of the hormone-dependent gene regulatory activity of h-TRβ1, we used partial proteolysis and anti-TR antibodies as probes to assess whether binding of T_3 to h-TRβ1 leads to any conformational changes. h-TRβ1 was treated with trypsin alone or

in the presence of T_3, or the thyroid hormone response element (TRE) or T_3 together with TREs. Without T_3, h-TRβ1 was completely digested by trypsin. Binding of TREs had no effect on the tryptic digestion pattern. However, T_3-bound h-TRβ1 became resistant to tryptic digestion and yielded trypsin-resistant peptide fragments with molecular weight of 28,000 and 24,000. Chymotryptic digestion also yielded a T_3-protected 24 Kd peptide fragment. Using anti-h-TRβ1 antibodies and amino acid sequencing, the 28 Kd fragment was identified to be Ser^{202}-Asp^{456}. The 24 Kd tryptic fragments were found to be Lys^{239}-Asp^{456} and Phe^{240}-Asp^{456}. The structural changes as a result of T_3 binding could serve as a transducing signal to affect the dissociation of TR homodimers from DNA. In our structure model, Lys^{201}, Trp^{234}, Arg^{238} and Lys^{239} are located on the amino terminal face of the barrel. They are on the same side of the DNA binding domain. Conceivably, the structural changes in the region between α0 and β-strand 1 and in the loop between helix 1 and β-strand 2 could directly or indirectly affect the interaction of DNA binding domain with DNA, thereby modulating the gene regulatory activity of h-TRβ1.

II. The molecular basis of generalized resistance to thyroid hormone (GRTH).

Generalized resistance to thyroid hormone (GRTH) is a syndrome characterized by refractoriness of the pituitary and peripheral tissues to the action of thyroid hormone and is usually transmitted in an autosomal dominant fashion. There is an inappropriately elevated level of thyrotropin stimulating hormone in the face of elevated levels of circulating free thyroid hormones, together with clinical features of hypo- and/or hyperthyroidism. The disease is caused by mutations in the ligand binding domain of TRβ1 which result in variable reductions in its affinity for T_3. The mutant TRβ allele inhibits the function of normal β- and α-receptor alleles via a dominant negative effect, thereby mediating the abnormal phenotypes. Kindreds with GRTH display differences in organ resistance within an individual as well as between kindreds harboring identical mutations.

The difficulty in correlating genotypes with the phenotypes stems from the complex molecular nature of the interactions of the thyroid hormone receptors. Not only are there four isoforms of the thyroid hormone receptors which are expressed in a tissue-specific and development-stage-specific manner, but they may also heterodimerize with multiple cellular partners, most prominently the retinoid X receptors (RXRs), to create unique transcriptional responses of T_3 target genes. Published examples of genotype-phenotype correlations include language abnormalities with mutations in exon 9 and a high ratio of mutant:normal TRβ mRNA during a period of bone resistance with growth retardation which was attenuated during teenage years with a concomitant improvement in growth rate. Neither the T_3-binding impairment as reflected by blood levels of thyroid hormones nor the location of mutations predicts a phenotype. To understand the molecular basis for the differential phenotypic expression, we have examined the interaction of mutant TRβ1 with cell-specific factors and with other nuclear receptors.

A. Cell-specific factors determine the dominant negative potencies of TRβ1 mutants.

We hypothesized that the ability of a mutant TR to exert different effects on T_3-responsive genes is dependent not only on TRE, but also on the cellular milieu in which it acts. To this end, we examined the effects of three naturally-occurring human mutant TRs on TREs, Lys (chicken

lysozyme gene TRE), ME (malic enzyme gene TRE) and GH (growth hormone gene TRE). The three mutants studies are kindreds PV (frame shift mutation), ED (A317T) and OK (M442V).

Transfection studies with wild-type TRβ1 in HeLa cells and NIH3T3 cells showed that T_3-induced transactivation varied markedly, suggesting that cell-specific factors contribute to these differences. Mutant TRs displayed the expected T_3-induced dose-responsiveness with the greatest difference occurring on the ME-TRE between the two cell types. In terms of the dominant negative effect, PV had the greatest inhibition of wild type TRβ1 function on the Lys-TRE in both cell-types at a 1:1 wild type:mutant receptor ratio. The magnitude of this dominant negative effect also varied between the cell types on the identical TRE. In gel-shift analyses, prominent heterodimers formed with both wild type and mutant TRs on Lys-TRE using HeLa nuclear extract, and much weaker ones with 3T3 nuclear extract, consistent with a ten-fold T_3-induced transactivation seen with wild type TRβ1 in HeLa cells compared to a five-fold T_3-stimulation in 3T3 cells. On the ME-TRE, heterodimers formed with wild type and mutant TRs with HeLa nuclear extract, but using 3T3 nuclear extract, both homodimers and heterodimers formed. Furthermore, the dominant negative effect of the various mutant receptors in 3T3 cells correlated well with the intensities of the homodimer bands. Thus, the strength of the dominant negative effect of mutant receptors is not only dependent on the nature of the mutation and the TRE but importantly, on the cell-type in which it acts as this determines the ability of wild type and mutant TRs to form specific homodimer and/or heterodimer bands. Our findings suggest that the heterogeneity of organ resistances seen in kindreds with GRTH is the result of the complex interplay of receptor-TRE interactions which itself is modulated by the nature of specific cellular factors.

B: The dominant negative potency of mutant human α1 and β1 thyroid hormone receptors, but not of h-TRβ1 and peroxisome proliferator-activated receptor, is T_3-response element selective.

While h-TRβ1mutants occur only in the families with GRTH syndromes, the ubiquitously expressed splicing variant h-TRα2 is known to inhibit normal h-TRα1 and h-TRβ1 transcriptional activity in transient transfection systems and is hence speculated to be a potential physiological modulator of thyroid hormone action. Although h-TRα2 was proposed to act through a similar mechanism as h-TRβ1 mutants, its inhibitory action has not been systematically compared to that of mutant h-TRβ1 on different TREs. Since the h-TRα2 protein is thought to antagonize thyroid hormone action mainly by competition for DNA-binding, other nuclear hormone receptors containing the same sequence of amino acids in their DNA-recognizing domain (P-box) might also be effective competitors for binding of h-TRβ1 to the AGGTCA half-site sequences. To address this possibility, we investigated the functional interaction of the recently cloned human peroxisome proliferator-activated receptor (hPPAR) with wild type h-TRβ1. The PPAR, which is activated by various agents, such as hypolipemic drugs (fibrates), arachidonic acid and fatty acids, has a P-box identical to that of the h-TRα and h-TRβ, as well as a conserved heterodimerization function with the RXR. An interaction between TRs and PPAR seemed physiologically plausible, since at a physiological level both of these signaling pathways are involved in the regulation of lipid metabolism.

We compared the dominant negative effect of mutant h-TRα1, h-TRβ1 and h-TRα2 on the three prototypic TRE, TRE-PAL (palindromic TRE), DR+4 (a direct repeat of half-site binding motif separated by 4 gaps) and TRE-LAP (an inverted Pal). The inhibitory effect of mutant h-TRα1

and β1 occurred only on TRE-LAP and to a minor degree on DR+4. This dominant negative effect was, therefore, TRE-selective at an equimolar ratios of wild type/mutant receptors. In contrast, the h-TRα2 inhibited TR action on all three TREs. Gel mobility shift experiments in the presence of T_3 showed increased binding of mutant h-TRα1 and β1 to TRE-LAP as compared to wild type h-TRs, thereby explaining their TRE-selective dominant negative potency. In contrast, an equal amounts of h-TRα2 protein did not bind to either of the three response elements, suggesting that the TRE-independent dominant negative action of h-TRα2 may not be due to competition for DNA-binding. Since thyroid hormones and peroxisome proliferators are hypothesized to regulate, at least in part, a similar subset of target genes involved in fatty acid metabolism, we investigated the interaction of TR and hPPAR on the three TREs. The PPAR was capable of inhibiting thyroid hormone induced trans-activation on the TREs tested, suggesting the possibility of cross-talk among the thyroid hormone and peroxisome proliferator-signaling pathways.

III. Thyroid hormone (TH) and tumorigenesis.

The role of TH in tumorigenesis has been controversial. However, increasing evidence has been presented to suggest that TH could play a vital role in cancer development. TH has been shown to modulate neoplastic transformation induced by x-rays, chemicals and viruses *in vitro*. TH was also found to have positive effects on breast cancer. It has also been reported that induced hypothyroidism inhibited the growth rate of Morris hepatoma and increased the survival rate of the tested animals. The administration of thyroxine reversed this inhibitory effect. To delineate the role of TRs in the process of oncogenic transformation, we studied the expression and regulation of TRs in nine human hepatoma cell lines.

The expression of TRα and TRβ genes was evaluated at both mRNA and protein levels. The expression of TRβ1 and TRα1 mRNAs is similar to those found in normal liver. However, the expression of TR proteins is receptor subtype- and cell type-dependent. TRα1 protein expresses similarly at a low level in each of the nine cell lines. In contrast, TRβ1 is overexpressed in Mahlavu, SK-Hep-1, and HA22T, moderately expressed in J5, J7, and J328 and is very low in HepG2, Hep3B, and PLC/PRF/5 cells. The overexpressed TRβ1 is down-regulated (2-3-fold) by T_3. The nine hepatoma cell lines arrested at different differential stages as indicated by the measurements of the known plasma protein markers. The degree of differentiation is found to be inversely correlated with the level of TRβ1 protein. Furthermore, T_3 was found to stimulate the proliferation of cell lines in which TRβ1 is overexpressed. These results suggest that TRβ1, not TRα1, is involved in the differentiation and proliferation of hepatoma cells. Our studies have shed new light in the understanding of the role of TRs in liver carcinogenesis.

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

PROJECT NUMBER
Z01 CB 08753-12 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

COOPERATING UNITS (If any)
Wilmington, DE; Cardiology Branch, NIH; Radiation Oncology Branch, NCI, NIH; Hoffman-La Roche, Nutley, N.J., Immunology Dept. Bristol-Myers Squibb, Seattle, WA; Centre de Recherche Hotel-Dieu de Montreal, Canada; (continued on next page)

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Biology

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
15.6	10.7	4.9

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

B

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

For the treatment of human cancer and AIDS, we have designed and produced several immunotoxins and fully recombinant toxins. Immunotoxin LMB-1 (composed of MAb B3 and a mutant form of *Pseudomonas* exotoxin, PE) is in clinical trials on patients with colon, breast, stomach and esophogeal cancers. LMB-7, an improved form of LMB-1, will enter trials in the same patient population in 1994. Other immunotoxins directed at the erbB2 oncoprotein (e23Fv-PE38KDEL) and at the IL2 receptor (anti-TacPE38KDEL) present on lymphomas and leukemias will enter the clinic in 1995. TP40 (TGFα fused to a 40,000 MW fragment of PE) is being evaluated in patients with bladder cancer. A recombinant toxin directed at the IL4 receptor present in kidney cancers has recently been made.

Recombinant immunotoxins with improved properties have been made which are: (1) smaller and therefore penetrate into tumors better; (2) less immunogenic and (3) more stable and easier to produce. Increased stability was achieved by using a combination of molecular modeling and genetic engineering to insert a disulfide bond at a conserved location in the framework region.

Site directed mutagenesis has been used to determine which surface residues in domains II and III of PE can be altered to cysteine or other amino acids without loss of activity and several sites were identified. These cysteine residues were used to make polyethylene glycol derivatives of PE. Even though large PEG molecules were added, almost full cytotoxicity was maintained.

Previously it was found that a large portion of domain Ib of PE could be deleted without loss of activity. We have now found that all of domain Ib (aa 365-395) and a portion of domain II can be deleted from single chain immunotoxins, if a glycine-rich connecting peptide is inserted between the recombinant domain II and domain III to keep the domains separated.

Other Personnel:

I. Benhar	Visiting Associate	LMB, NCI
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P. Chowdhury	Visiting Fellow	LMB, NCI
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L. Pai	Senior Staff Fellow	LMB, NCI
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K. Webber	Senior Staff Fellow	LMB, NCI
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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 is in collaboration with M. M. Gottesman, LCB concerns the molecular basis of multidrug resistance in cancer and the development of *MDR*1 containing vectors for gene therapy.

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 used to date is a 38,000 molecular weight molecule termed PE38. It 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 which is used to couple LysPE38 to antibodies. The principle monoclonal antibody under development is MAb B3 which reacts with a carbohydrate antigen present on many human cancers including cancers of the colon, breast, ovary, lung, stomach, esophagus, bladder and prostate. The other type of immunotoxin is wholly recombinant and consists of the Fv portion of an antibody fused to PE38.

Clinical Studies:

Lee Pai, in collaboration with R. Wittes of the Medicine Branch, NCI, is conducting a phase I clinical trial with LMB-1 (B3-LysPE38), an immunotoxin in which MAb B3 is coupled to PE38. 19 patients with advanced breast, colon and other cancers containing the B3 antigen have been entered at doses from 10 μg/kg/day to 100 μg/kg/day given IV on days 1, 3, 5. Capillary leak syndrome is the major side effect. Pharmacokinetic studies show that in most patients, LMB-1 is cleared relatively slowly with a half-life of about 6-10 hours. 16/18 patients developed anti-PE antibodies after one course of treatment. Antitumor activity was observed in 2/18 patients. Dose escalation will continue until a maximum tolerated dose is established.

LMB-7 is a recombinant immunotoxin in which the Fv fragment of MAb B3 in a single-chain form is fused to PE38. LMB-7 is more active than LMB-1 on cultured cell lines and in mice bearing human tumors as xenografts. LMB-7 has completed preclinical development and is ready for a phase I clinical trial. The agent has been produced by Boehringer Mannheim under a CRADA. The clinical trial on patients with advanced antigen positive cancers will begin in the summer of 1994.

MAb B3 has also been radiolabeled with ^{111}In and ^{88}Y and shown to localize to xenografts bearing the B3 antigen. L. Pai in collaboration with J. Carrasquillo (Nuclear Medicine) is beginning a phase I imaging and radiotherapy trial with MAb B3 in patients with advanced cancers containing the B3 antigen.

Preclinical Studies:

Single-chain immunotoxins (SCIT) that cause complete regression of antigen positive cancers growing in nude mice have been made with two other antibodies. One is an antibody to the erbB2 oncoprotein. This agent (e23(Fv)-PE38KDEL) is being developed under a CRADA with Oncologix. This immunotoxin has a mutant carboxyl terminus in which REDLK of PE is changed to KDEL to increase potency. It is scheduled to enter clinical trials in erbB2 positive cancers in 1995.

R. Kreitman is completing preclinical evaluation of immunotoxin anti-Tac(Fv)-PE38KDEL directed at the IL2 receptor present on leukemias and lymphomas. A pre IND meeting with the FDA is scheduled and the clinical drug lot is being prepared. The MTD in monkeys is about 300 μg/kg. Good antitumor activity in mice observed at 50 μg/kg. Anti-Tac(Fv) KDEL should enter a phase I clinical trial in 1995 supervised by R. Kreitman in collaboration with T. Waldmann.

TP40 is a recombinant molecule in which TGFα is fused to a 40,000 molecular weight form of PE. TP40 was licensed to Merck who completed in 1993 a phase I trial in which TP40 was administered to patients with superficial bladder cancer weekly for 6 weeks by the intravesicular route. No toxicities and several clinical responses were noted, one response lasting >6 months. A second trial in bladder cancer as well as trials administering the agent intravenously for breast, ovarian and lung cancer are planned for 1995. In previous studies with human xenografts in mice, TP40 has displayed antitumor activity against an epidermoid carcinoma, a glioblastoma, and a prostatic carcinoma; all these contain large numbers of EGF receptors.

Improved Recombinant Immunotoxins:

To be effective as an anticancer agent, an immunotoxin must: (1) selectively bind to cancer cells, (2) be small enough to rapidly penetrate from the circulation into cancers, (3) be stable and easy to produce in large amounts and (4) have low immunogenicity.

Size:

The current generation of single-chain immunotoxins are much smaller (Mr 66 kDa) than conventional immunotoxins (Mr ~200 kDa). To make even smaller molecules, A. Kihara and C. Kuan have identified sequences in domains II and Ib of PE that can be deleted. These studies have led to a new B3 containing SCIT, B3(Fv)-PE33, which has a MW of ~62 kDa. A. Kihara has also made new forms of TP40 which are about 20% smaller, up to 1000 times more active in tissue culture and show good antitumor activity in mice.

Improved Stability:

Some SCITs and many single-chain Fvs are difficult to produce and purify, because they are unstable and readily form aggregates. To overcome this problem, B. K. Lee and S. H. Jung designed a new type of Fv molecule that is stabilized by a disulfide bond located at a specific site that is highly conserved and therefore present in the framework region of all V_H and V_L domains. Using their design, U. Brinkmann and Y. Reiter have made disulfide linked Fv immunotoxins with three different antibodies (B3, e23, anti-Tac) and found that all three make active recombinant immunotoxins which are easily produced and very stable. In addition, the dsFv immunotoxin made with MAb e23 is more active as an antitumor agent than its SCIT counterpart because of improved antigen binding.

Immunogenicity:

To determine B cell epitopes present in the PE portion of LMB-1 and LMB-7, D. Roscoe used a panel of 120 overlapping peptides containing sequences from PE38 to study the reactivity of sera from humans treated with LMB-1 and from monkeys treated with LMB-1 and LMB-7. Six major epitopes were discovered. She has also isolated a panel of 14 mouse monoclonal antibodies that bind to domains II, Ib or III of PE. Our goal is to use information about epitopes in the PE portion of LMB-7 to make mutant LMB-7 molecules in which the epitopes are deleted, eliminated by site-directed mutagenesis or "covered up" by derivitization with polyethylene glycol.

I. Benhar and C. Kuan have carried out mutagenesis studies on domains III and II respectively to determine which surface residues can be deleted or mutated without loss of function. They also used these molecules, in collaboration with Q. Wang, to make specific polyethylene glycol (PEG) derivatives of LMB-7 or PE. Several of these derivatives retained very high cytotoxic activity despite the presence of bulky PEG moieties attached to LMB-7 by either thioether or disulfide bonds. In another approach to reduce the immunogenicity of LMB-7, I. Benhar humanized its Fv domain. By making these molecules as immunotoxins and expressing them in *E. coli,* several different mutant molecules can be produced and tested simultaneously and quickly. This strategy is a convenient way of humanizing the Fv domains of other antibodies.

Vascular Leak Syndrome:

In mice and monkeys, dose limiting toxicity with LMB-1 occurs at 3-4 mg/kg and is due to liver damage (mice) and stomach damage (monkeys). In humans, vascular leak syndrome (VLS) develops at much lower doses limiting the amount of LMB-1 that can be given to patients. To investigate the basis of VLS, C. Kuan studied the cytotoxic activities of LMB-1, LMB-7 and non-B3 containing immunotoxins on vascular endothelial cells. He found that some of these cells contain small amounts of the B3 antigen. Its presence results in cell death at very high LMB-7 and LMB-1 concentrations. These results suggest that because LMB-1 has a long half-life in the circulation of humans (~8 hrs), endothelial cells are preferentially exposed to high concentrations of LMB-1 for prolonged periods before it reaches tumor cells and therefore these endothelial cells are damaged allowing proteins to leak out of the vascular system. We hypothesize that wholly recombinant molecules that are small (MW ~66 kDa) and rapidly leave the circulation ($T_{1/2}$ 20-40 min) should have less of this dose limiting side effect. Also immunotoxins targeted to other "tumor" antigens should not produce this side effect. Clinical studies to begin in 1994 will clarify this issue.

Growth Factor Toxins:

Several IL4-PE molecules have been previously produced and shown to be active on hematologic and solid tumors, but these have relatively low activity. R. Kreitman has shown that the low activity is due to impaired receptor binding, because the toxin is fused to residues at the carboxyl terminus of IL4 essential for binding to the IL4 receptor. To circumvent this, he has made circularly permuted forms of IL4 in which the amino end is joined to the carboxyl end and new termini are created in other regions of the molecule. IL4-toxins made with circularly permuted IL4 have markedly improved binding and cytotoxicity on target cells and are candidates for preclinical development.

IL6-PE is toxic to myeloma cells which often contain increased numbers of IL6 receptors. It is being developed by R. Kreitman, in collaboration with B. Barlogie, University of Arkansas, as a bone marrow purging agent for use in bone marrow transplantation in patients with multiple myeloma.

Heregulins are ligands for some members of the EGF receptor family. A. Kihara has cloned four different forms of heregulin and used these to make chimeric toxins directed at cells overexpressing erbB3 or erbB4 receptors. These agents are also active in tumor bearing mice.

E. Mesri and R. Kreitman have used heparin binding EGF or its heparin binding domain alone to make chimeric toxins for the study of receptors that bind these ligands.

Radiolabeled Antibodies:

Taking advantage of the stability of ds(Fv) immunotoxins, K. Webber developed methods for preparing a ds(Fv) form of anti-Tac which is more stable than the single-chain form. Anti-Tac-ds(Fv) can be iodinated with ^{125}I with full preservation of binding activity. In mice, this agent rapidly concentrates in antigen positive tumors. An imaging trial in patients with adult T-cell leukemia and other IL2 receptor containing malignancies is being planned.

Phage display with ds(Fv):

U. Brinkmann and P. Chowdhury have taken advantage of the stability of ds(Fv)s to make phage display libraries with them. Using anti-Tac as a model antibody, a dsFv phage was made that binds to the α subunit of the IL2 receptor. This phage is much more resistant to inactivation by elevated temperature than its single-chain counterpart. Also, a dsFv phage library was constructed from mouse spleen RNA and is currently being characterized.

AIDS:

CD4-PE40 is a recombinant toxin that binds to gp120 or the surface of HIV infected cells and kills these cells. CD4-PE40 has been evaluated in phase I trials for the treatment of AIDS but is not useful because in humans with AIDS it produces severe liver toxicity, which was not observed in preclinical studies in monkeys and rodents. We have begun in investigate that in human blood CD4-PE40 may form a complex with gp120 that directs it to the liver. We are also studying the use of immunotoxins to target gp41 or gp120 by making single chain immunotoxins directed at the antigens. Several types of single chain immunotoxins have been made with MAb 98-6 directed at gp41. These are active on transfected CV1 cells expressing gp41 and are being tested on human T cells infected with HIV. A SCIT directed at gp120 is currently being made.

CRADA
Contractor: Oncologix
CRADA Number: CACR 0122
Title: Therapy of Cancers Expressing erbB2 Oncogene
Date Initiated: 11/14/91
Funding level: $70,000
Objective: The objective of this CRADA is to improve the activity and other properties of the immunotoxin e23(Fv)PE38KDEL. To accomplish this, we made a disulfide-linked Fv form of this molecule and showed it is more active than the single chain form of the immunotoxin in cell culture. The increase in activity is due to better binding to the erbB2 protein and the increased activity in tissue culture also translates into incrased antitumor activity in animals.

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

PROJECT NUMBER
Z01 CB 08754-11 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
13.5	11.5	2.0

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

B

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

Our work has emphasized two aspects of the molecular biology of multidrug resistance (MDR) in cancer cells: 1) the mechanism of action of the multidrug transporter also known as P-glycoprotein (PGP), the product of the *MDR*1 gene; and 2) the use of vectors encoding the *MDR*1 gene to confer selective advantage on cultured cells and in cells in intact animals and patients. Mechanistic studies have involved biochemical purification of both naturally occurring (from MDR cell lines) and recombinant (from baculovirus) PGP. PGP purified to near homogeneity demonstrates drug-dependent ATPase activity and transport activity when reconstituted in lipid vesicles. Phosphorylation of PGP is not necessary for its activity, as shown by recombinant expression vectors in which all of the known phosphorylation sites on PGP have been deleted. A new kinase present in plasma membranes of cultured cells which phosphorylates PGP has been discovered and purified from cultured cells. To develop a model system for studying the effect of specific mutations on ATPase and substrate binding activities of PGP, the human *MDR*1 cDNA has been expressed in the yeast *Saccharomyces cerevisiae*, resulting in resistance to several different anti-cancer drugs. For gene therapy of cancers, *MDR*1-encoding retroviral vectors which confer MDR on the bone marrow of mice have been developed. One bicistronic vector in which the Herpes simplex virus thymidine kinase (HSK-TK) gene is under control of an internal ribosome entry site (IRES) present on the same mRNA which encodes *MDR*1 can be used to kill cancer cells which have been inadvertently transduced with the *MDR*1 gene during gene therapy to protect bone marrow against the toxic effects of chemotherapy. Other vectors for gene therapy of inborn errors of metabolism carry the *MDR*1 cDNA as a selectable marker and the non-selectable cDNAs for glucocerebrosidase (Gaucher disease), alpha galactosidase (Fabry disease) and the 91 kDa subunit of the NADPH oxidase complex (chronic granulomatous disease).

Other Professional Personnel:

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Major Findings:

1. Reconstitution of transport with purified PGP: P-glycoprotein (PGP) has been purified to near homogeneity from highly multidrug resistant (MDR) KB-V1 cells after extraction with octyl glucoside and fractionation on DEAE Sepharose CL6-B and wheat germ agglutinin columns. This purified protein in detergent shows some basal ATPase activity, but when reconstituted into liposomes prepared from *E. coli* lipids, cholesterol, phosphatidylserine and phosphatidylcholines, displays both basal and drug-stimulated ATPase activity (15-30) μmole of phosphate hydrolyzed per mg of protein). When reconstitution is performed under conditions which allow formation of proteoliposomes with reduced lipid to protein ratio, ATP-dependent transport of vinblastine can be clearly demonstrated. These studies provide biochemical evidence that PGP itself is sufficient for drug transport. To obtain larger amounts of material suitable for structural analysis and analysis of the mechanism of action of PGP, the human *MDR*1 cDNA was engineered to include a nucleotide encoding a polyHis (6) track at the carboxy-terminus or in the linker region connecting the two homologous halves, and inserted into a baculovirus vector. Protein encoded by the baculovirus vector with the polyHis track at its carboxy-terminus, expressed in insect Sf9 cells, demonstrates drug-dependent ATPase activity in crude membrane preparations. PGP-His(6) can be purified on Ni-NTA columns.

2. Phosphorylation is not essential for function of PGP: Indirect evidence has suggested that phosphorylation of PGP by protein kinase C (PKC) may activate its transport function. There are five sites of phosphorylation by PKC in PGP in the region connecting the two halves of PGP (Ser 661, Ser 667, Ser 671, Ser 675, Ser 683), of which three (Ser 661, Ser 667, Ser 683) appear to be major sites phosphorylated both *in vitro* and *in vivo*. Chimeric peptide fragments in which mutant and wild-type linker peptides from PGP were linked to glutathione-S-transferase for purification on glutathione affinity columns were shown to be phosphorylated *in vitro* by PKC as expected from results using intact PGP. Mutant peptides lacking major sites of phosphorylation were poorly phosphorylated, as expected. A novel kinase has been purified to near homogeneity from KB-V1 plasma membranes which is a potent kinase for PGP *in vitro*. This kinase is not Ca-dependent, has a molecular weight of approximately 60,000, and is quite active in phosphorylating the GST-PGP peptides containing known phosphorylation sites. Of the three major phosphorylation sites on PGP, only two (Ser 661, Ser 667) are phosphorylated by the V1 kinase. The regulation and function of this novel kinase are not yet known, but its colocalization with PGP in the plasma membrane and its higher specific activity than

PKC versus PGP and its peptides, suggest it may play a major role in determining the extent of phosphorylation of PGP *in vivo*. To determine whether any of the five potential phosphorylation sites in the connecting region of PGP are essential for function, all five were replaced with Ala (to eliminate phosphorylation) or Asp (to preserve negative charge). Both constructs were inserted into pHaMDR expression vectors, and both conferred multidrug resistance indistinguishable in our initial analysis from wild-type PGP. The five Ala-PGP showed no evidence of phosphorylation of any other sites *in vitro* by PKC, PKA or VI kinase or *in vivo*. These results argue strongly that we have identified the major sites of phosphorylation of PGP, and that phosphorylation of these sites is not essential for function of PGP as a multidrug transporter, but do not rule out a role for phosphorylation in modulating an as yet unidentified function of PGP.

3. Molecular genetic studies on ATPase activity and substrate utilization: In addition to the biochemical approach described above, we have attempted to study the molecular requirements for ATPase activity by forming chimeras between PGP encoded by *MDR*1 and other members of the ATP binding cassette (ABC) superfamily of transporters. Initial studies show that both amino- and carboxy-terminal ATP sites from *MDR*2 (a related transporter expressed in liver which is essential for release of phosphatidyl-choline into bile) will substitute for the ATP sites in *MDR*1. However, ATP sites from CFTR do not form functional chimeras. Analysis of cell surface expression of chimeric PGPs indicates that the major reason for functional inactivation of chimeras is their failure to be expressed on the cell surface, either because they are improperly processed in the ER or Golgi, or degraded prior to reaching the cell surface. Using iodo-deoxyforskolin as an affinity label, the major substrate interaction sites in PGP have been localized to transmembrane domains (TMs) 5 and 6, TMs 11 and 12, and the extracellular and intracellular sequences associated with these TMs. A bicistronic expression vector (see below) using methotrexate resistance as a selectable marker, and *MDR*1 as the cDNA whose function is being analyzed, has been designed to allow introduction of random mutations into the substrate binding sites of *MDR*1 to define residues essential for substrate binding and specificity. The expression of the human *MDR*1 gene in *Saccharomyces cerevisiae* defective in the ERG6 gene (lack of ergosterol in the plasma membrane in ERG6 mutants sensitizes yeast to anti-cancer drugs and other cytotoxic agents which are MDR substrates) results in MDR. This heterologous expression system can be used to test the effect of various mutations in the ATP and substrate utilization sites.

4. *MDR*1 vectors for gene therapy: We have previously shown that expression of the human *MDR*1 gene in the bone marrow of transgenic mice under control of a chicken actin promoter results in resistance of bone marrow to the toxic effects of anti-cancer drugs. After several generations of inbreeding, the *MDR*1 transgenic mice no longer express detectable levels of PGP in their marrow. We have constructed three new lines of transgenic mice carrying the *MDR*1 gene under control of mouse and human immunoglobulin heavy chain upstream sequences. In one such line, human PGP mRNA is found in peripheral blood cells, and in bone marrow, spleen, thymus, and intestine, consistent with expression in lymphocytes. Studies are in progress to determine whether B lymphocytes expressing the human *MDR*1 cDNA are resistant to the cytotoxic effects of taxol and daunorubicin. Additional studies indicate that transduction of murine bone marrow with *MDR*1 retroviral vectors results in a selective advantage of the transduced cells over non-transduced cells in bone marrow. These observations have resulted in two new approaches to gene therapy which exploit our ability to select for cells expressing the human *MDR*1 cDNA. In the first approach, the *MDR*1 cDNA in a retroviral vector is used to confer MDR to bone marrow in patients undergoing intensive chemotherapy. We will be collaborating on two clinical trials (at NIH and at Columbia University Medical Center) to test this approach in breast cancer patients undergoing autologous bone marrow transplantation. The clinical trial at Columbia is part of a CRADA with GENETIX PHARMACEUTICALS CORPORATION (CACR-0150) initiated 11/08/93 and entitled "MDR Gene Expression in Hematopoietic Stem Cells." We have already developed one such vector in which both *MDR*1 and the Herpes simplex virus thymidine kinase (HSV-TK) gene are expressed in a single bicistronic expression vector. In this vector system, the *MDR*1 gene is under

control of the retroviral LTR promoter, and the HSV-TK gene, on the same message, is independently translated because it is downstream from an internal ribosome entry site (IRES). In this system, *MDR*1-expressing cells also express HSV-TK; this expression sensitizes recipient cells to ganciclovir and would allow the elimination of any cancer cells in the bone marrow which were inadvertently transduced by the *MDR*1 retroviral vector. The second approach involves similar bicistronic vectors in which *MDR*1 is the selectable gene, and the non-selectable gene may be any one of a variety of cDNAs encoding proteins defective in inborn errors of metabolism. To date, with the help and collaboration of Roscoe Brady, Edward Ginns and Harry Malech, we have constructed such vectors for glucocerebrosidase (defective in Gaucher disease), alpha-galactosidase (defective in Fabry disease), and the 91 kDa subunit of NADPH oxidase complex (defective in chronic granulomatous disease). All show expression of the non-selectable cDNA when cells are selected for *MDR*1 expression *in vitro*. Several successful approaches to transduction of murine bone marrow stem cells with *MDR*1 retroviral vectors are under development, including *ex vivo* selection and growth of stem cells in medium containing stem cell factor, IL-3, and IL-6, retroviral transduction of whole bone marrow or fetal hepatic hematopoietic elements, and liposome mediated transfer of retroviral DNA to bone marrow cells.

5. Mechanism of high level cisplatin resistance in cultured human KB adenocarcinoma and hepatoma cells: As detected on 2D gels, there are many alterations in expression of proteins from KB adenocarcinoma and BEL-7404 hepatoma cells selected for high level (approximately 100-fold) resistance to cisplatin. However, one prominent change seen in both KB and hepatoma cells is the overexpression of a protein of molecular weight 52-56 kDa. This protein has been isolated and microsequenced and found to be identical to the mitochondrial matrix heat shock protein hsp60. cDNA probes for hsp60 detect overexpression of mRNA in the highly cisplatin resistant cell lines. In collaborative studies with Dr. Thomas Hamilton (Fox Chase Cancer Center) and Shin-ichi Akiyama (Kagoshima University, Institute of Cancer Research), the highly resistant human cell lines developed in our laboratory have been shown to have decreased accumulation of cisplatin in cells and decreased platination of DNA. The relationship between decreased drug accumulation and increased expression of hsp60 is under investigation, but is as yet unexplained.

CRADA Information:

Contractor: GENETIX PHARMACEUTICALS CORPORATION
CRADA Number: CACR-0150
Date Contract Initiated: 11/08/93

Publications:

Roninson I, Pastan I, Gottesman, MM. US Patent 5,206,352: Compositions for Clones Containing DNA Sequences Associated with Multidrug Resistance in Human Cells, April 27, 1993.

Gottesman MM, Mickisch, GH, Pastan I. *In vivo* models of P-glycoprotein-mediated multidrug resistance. In: Goldstein LJ, Ozols R, eds. Anticancer drug resistance. Advances in molecular and clinical research; ch 6, Norwell: Kluwer Academic;1993:103-24.

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

PROJECT NUMBER
Z01 CB 08756-07 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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Others:	C. Jhappan	Senior Staff Fellow	LMB, NCI
	H. Takayama	Visiting Fellow	LMB, NCI
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LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Genetics Unit

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

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

B

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

Transgenic technology is being used to examine the role of specific ligands and receptors in growth and differentiation *in vivo* and to establish useful and novel animal models to aid in the study of pathogenesis in human disease, including cancer. Transforming growth factor α (TGFα) stimulates a variety of biological responses by binding to the epidermal growth factor receptor (EGFR) and activating its tyrosine kinase, and has been implicated in human cancer. Overexpression of TGFα in transgenic mice induces a high incidence of malignant lesions in the liver and mammary gland. TGFα acts in a synergistic fashion with the c-Myc nuclear oncoprotein in hepatic and mammary tumorigenesis in bitransgenic mice overexpressing both transgenes. TGFα collaborates with diverse chemical agents in carcinogenesis of the liver and skin, including genotoxic initiators and nongenotoxic promoters, demonstrating that TGFα possesses diverse oncogenic potential. Constitutive TGFα-driven EGFR stimulation can substitute functionally for mutational activation of the c-Ha-*ras* proto-oncogene in skin tumorigenesis. In contrast to its role as an oncogenic agent in the liver, breast, and skin, TGFα overexpression disrupts differentiation pathways in the stomach, pancreas, and salivary glands, resulting in the development of dramatic metaplastic lesions and a depletion of specific mature cell types.

We continue to study mice bearing transgenes encoding other relevant growth and differentiation factors. Transgenic mice made with an activated form of an EGF-related gene, *int-3*, develop severe hyperplastic and developmental lesions of multiple secretory glands and cancer of the salivary and mammary glands. Bitransgenic mice overexpressing activated *int-3* and TGFα demonstrate highly accelerated mammary tumorigenesis, indicating that these two factors can collaborate in breast cancer. Mice overexpressing a transforming growth factor β1 (TGFβ1) transgene in the pregnant mammary gland were unable to lactate due to inhibition of the lobuloalveolar development and suppression of endogenous milk production. Transplantation studies suggest that TGFβ1 induces this phenotype by causing premature senescence of alveolar stem cells, providing a useful model to study differentiation and programmed cell death in the mammary gland. Moreover, TGFβ1 inhibits TGFα-induced mammary tumorigenesis in TGFα/TGFβ1 bitransgenic mice.

Major Findings:

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

Overexpression of TGFα and the EGF receptor can transform cells in culture. To determine the *in vivo* consequences of perturbing EGF receptor signal transduction pathways, we generated mice bearing TGFα and EGF receptor transgenes. In one series of experiments, a DNA fragment containing a human TGFα cDNA driven by the mouse metallothionein-1 promoter was microinjected into mouse embryos. Mice expressed this transgene in multiple tissue types including the liver, pancreas, stomach, breast, and skin. Elevated levels of TGFα were detected in the blood and urine of transgenic mice, as has been shown in cancer patients. TGFα transgenic mice developed many remarkable lesions, making them useful as animal models for a number of important human diseases.

Approximately half of all multiparous TGFα transgenic female mice developed multifocal, transplantable mammary adenomas and adenocarcinomas by about 9 months of age. Tumors were rare in virgin animals, indicating an important role for pregnancy-associated hormones in TGFα-induced breast cancer. TGFα transgene expression was greatly enhanced in the majority of premalignant and malignant lesions of the mammary gland, suggesting that mammary epithelial cells that overexpress TGFα gain a selective growth advantage.

One year old male, but not female, transgenic mice exhibited an ~75% incidence of benign and malignant liver tumors, most of which greatly overexpressed the TGFα transgene. Ovariectomy stimulated hepatocarcinogenesis in female transgenic mice while castration was protective in male transgenic animals. This identified sex hormones as important factors in the development of these liver tumors. To identify other collaborating factors associated with TGFα-induced oncogenesis, liver tumors from transgenic animals were characterized at the molecular level. Mutations in the *ras* proto-oncogenes were not associated with hepatocarcinogenesis. In contrast, expression of endogenous insulin-like growth factor II and c-Myc was frequently greater in liver tumors.

The ability of c-Myc to cooperate with TGFα in liver and mammary tumor development was tested directly by mating our TGFα transgenic mice with transgenic mice in which the c-*myc* proto-oncogene was overexpressed either in the liver (in collaboration with Dr. Snorri Thorgeirsson) or the mammary gland (in collaboration with Dr. Robert Dickson). As a consequence of overexpressing both TGFα and c-Myc, highly malignant tumors developed at an accelerated rate in both the liver and the mammary gland of bitransgenic mice. Interestingly, hepatic and mammary tumors developed in TGFα/c-Myc mice

irrespective of their sex, overcoming limitations associated with sex hormones in both tissue types. Moreover, the salivary glands, which exhibited only benign hyperplastic lesions in single TGFα transgenic mice, developed a new phenotype in TGFα/c-Myc bitransgenic mice: frank tumors. These results illustrate the genetic power of the transgenic mouse technology and demonstrate clearly that potent growth factors and nuclear proto-oncogenes can constitute strong collaborative agents in oncogenesis.

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

Transgenic mice have also been useful as a source of genetically altered cells for *in vitro* culture. We have, in collaboration with Dr. Nelson Fausto, established two apparently normal hepatocyte lines from the livers of TGFα transgenic mice without the aid of viral oncogenes. These unique lines are nontumorigenic and possess a multitude of morphological and biochemical traits associated with the fully differentiated state. These cell lines should prove to be valuable models for the study of hepatocyte growth and differentiation.

To elucidate molecular mechanisms by which TGFα induces premalignant and malignant lesions, we chose chemical carcinogenesis of transgenic mouse skin as an experimental model system. The tumor initiator 7,12-dimethylbenz[*a*]anthracene (DMBA), shown previously to induce in normal mouse skin, in concert with a tumor promoter, papillomas containing specific c-Ha-*ras* mutations. DMBA treatment alone induced the formation of a variety of benign and malignant squamous and sebaceous glandular lesions in virtually all TGFα transgenic mice but not in control mice. Interestingly, the majority of skin tumors did not contain c-Ha-*ras* mutations but demonstrated a 10- to 20-fold enhanced expression of the TGFα transgene. In contrast, in skin tumors that harbored a mutant c-Ha-*ras* gene, TGFα transgene expression was always relatively low. These results suggest that strong constitutive EGFR stimulation, through TGFα overexpression, can substitute functionally for mutational activation of c-Ha-*ras* in skin tumorigenesis. Moreover, because c-Ha-*ras* mutational activation could not induce skin tumors without the TGFα transgene, simultaneous stimulation of an EGFR-mediated c-Ha-Ras-independent pathway appears to be required for tumor development as well. Results from our analysis of *ras* in liver tumors from TGFα transgenic mice suggest that these conclusions may apply to other tissues as well.

TGFα also induced a florid ductular metaplasia of the transgenic exocrine pancreas accompanied by severe interstitial fibrosis. 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 that are found in many diseases of the human pancreas. The TGFα transgenic mouse may, therefore, represent a valuable animal model for the study of the development and treatment of human pancreatic disease. Significantly, this redifferentiation process and the formation of tubular complexes were also seen in the transgenic salivary glands, which was found to overexpress TGFα.

TGFα overexpression also caused dramatic structural and functional lesions of the glandular stomach that resemble Ménétrier's disease in human patients. 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 depleted. In fact, TGFα induced a widespread cellular disorganization of the gastric mucosa in which the surface mucous cell population in the gastric pit was expanded dramatically at the expense of the glanular base. RNA blot and *in situ* hybridization demonstrated that functional parietal and chief cells were specifically lost from the mucosa, coinciding in time with activation of TGFα transgene expression in the neonatal stomach. These findings suggest that TGFα participates in normal regulation of proliferation and differentiation during epithelial cell renewal. Moreover, TGFα may contribute to the pathogenesis of related human stomach disorders such as Ménétrier's disease.

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 specific cell types 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 may constitute a useful model system for studying male infertility.

Transgenic mice are also being used to study related growth and differentiation factors. The *int-3* gene was identified through its participation in mouse mammary gland oncogenesis. The mouse mammary tumor virus (MMTV) has been shown by Dr. Robert Callahan to integrate into and activate the *int-3* gene, which contains repeated sequences similar to the yeast *cdc10* cell cycle "start" protein and to EGF. We recreated this integration event in transgenic mice harboring a DNA fragment containing the activated *int-3* gene under the transcriptional control of the MMTV long terminal repeat. *Int-3* transgenic mice developed multifocal, poorly differentiated mammary and salivary adenocarcinomas between 2 and 7 months of age. Mammary glands were arrested in development and lactation-deficient in all female *int-3* mice. Hyperplastic, immature ductule cells were observed in all transgenic salivary glands and in numerous glands of the head and neck. In addition, all male transgenic founders were sterile due to severe hyperplasia of the epididymis. All affected tissues expressed the *int-3* transgene. These findings demonstrate *in vivo* that expression of the activated *int-3* gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia. Significantly, bitransgenic mice expressing both the TGFα and *int-3* transgenes exhibited highly accelerated mammary gland tumorigenesis, suggesting that these two factors can function synergistically in the development of breast cancer.

Transforming growth factor β1 (TGFβ1) is characterized by diverse and highly potent cell-specific effects. These can either complement or oppose proliferative activities associated with TGFα depending on the cell type. To determine the *in vivo* effects of inappropriate TGFβ1 expression and to elucidate potential interactive mechanisms between TGFβ1 and TGFα, we have made transgenic mice expressing a TGFβ1 gene in the pregnant mammary gland by virtue of the whey-acidic protein gene promoter. Female transgenic mice overexpressing TGFβ1 in the mammary gland experienced inhibited

lobuloalveolar formation and suppressed milk production, supporting an *in vivo* role for TGFβ1 in regulating the development and function of the mammary gland. Transplantation studies, in collaboration with Dr. Gilbert Smith, suggest that TGFβ1 induced this phenotype by stimulating premature senescence of alveolar stem cells, providing a useful model system for differentiation and programmed cell death of mammary tissue. To determine if TGFβ1 is capable of suppressing the development of mammary tumors induced by TGFα, we have generated and are presently analyzing female mice that are bitransgenic for TGFβ1 and TGFα. Preliminary results indicate that TGFβ1 can effectively inhibit the formation of hyperplastic and malignant lesions of the breast associated with TGFα overexpression.

Previously, we had isolated and characterized *in vitro* the EGF receptor gene promoter with the intention of elucidating transcriptional regulatory mechanisms. To study promoter function in a more relevant environment, fragments of genomic DNA sequences containing EGF receptor gene *cis*-acting elements, known to be required for optimal activity in transfected cultured cells, were used to drive expression of the chloramphenical acetyltransferase (CAT) reporter gene in transgenic mice. Unexpectedly, in all expressing lines of transgenic mice the EGF receptor gene promoter was active primarily in the thymus. Cell separation techniques showed that the promoter was not active in thymocytes but was instead expressed in the thymic epithelial cell-containing stromal tissue. Our results raise the possibility that the EGF receptor may play some role in thymic epithelial cell function and/or thymocyte selection. We plan to use this novel promoter to target expression of various transgenes, including oncogenes, to the thymic epithelium.

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

PROJECT NUMBER
Z01 CB 08757-07 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

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

B

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

Interactions of *Pseudomonas* Exotoxin (PE) with mammalian cells were investigated. Since PE binds LRP, the role of this receptor in mediating PE toxicity was studied. CHO cells were mutagenized, PE resistance clones were selected and analyzed for possible alterations in LRP expression. To enrich for receptor-specific defects, only cells that retained their sensitivity to DT were retained. Analysis of seven independent lines revealed two classes of mutants: one class, with two members, had no detectable LRP while the second class, with five members, expressed LRP but at a reduced level. The single chain LRP in the second class was not processed efficiently into heavy and light chains. One cell line from each group was studied in detail. The 13-5-1 line, with no LRP was 100-fold resistant to PE while the 14-2-1 line, which reduced amounts of LRP, was 10-fold resistant. Resistance to PE was related to LRP expression since neither line was resistant to a chimeric toxin composed of transferrin linked to domains II and III of PE. Results suggest that LRP is the surface receptor used in the pathway of PE-mediated toxicity. Domain I of PE mediates toxin binding to the heavy chain of LRP. By replacing domain I with other LRP-binding ligands, the relationship of binding to toxicity can be investigated. Therefore, gene fusions were made between either PE or diphtheria toxin (DT) and the LRP receptor associated protein (RAP). Likewise, a DT PAI-I fusion was made. Results indicated that fusions proteins were less active than native PE. After receptor-bound toxin is internalized, it is cleaved by a cellular protease. PE, PEala281 and DT were cleaved by a furin-like enzyme prepared from beef liver. The beef protease and recombinant furin exhibited identical enzymatic activities. Immunodepletion of the beef protease with anti-furin antibodies removed all toxin-cleavage activity. An *in vivo* role for furin-mediated cleavage of PE is supported by data showing that arginines at P1 and P4 are important for both toxicity and cleavage and that furin-nicked PE has increased toxicity compared to intact toxin. The proteolytic cleavage of TGFαPE38 was also studied. Initially, two mutant forms, TGFαPE38PEgly276 and TGFαPE38gly279 were constructed. These mutants proved less toxic than TGFαPE38 and were refractory to furin-mediated cleavage, suggesting that the need for proteolytic processing of this chimeric toxin was similar to that of native PE. Immunotoxins directed to surface antigens expressed on myeloid leukemia cells were made and had ID50 values in the range of 20-200 ng/ml on K562 cells.

Major Findings:

Toxin-mediated selection and screening yields mutant cells with altered expression of LRP

The alpha 2-Macroglobulin Receptor/Low Density Lipoprotein Like Receptor Protein (LRP) plays an important role in the clearance of protease-inhibitor complexes and various ligands associated with lipid metabolism. While the regulation of receptor function is poorly understood, the addition of high concentrations of the 39 kD receptor-associated protein (RAP) to cells inhibits the binding and or uptake of many of these ligands. Previously, we (Kounnas et al, J Biol Chem 267:420-3, 1992) showed that *Pseudomonas* exotoxin (PE) could bind to the LRP when it was immobilized on nitrocellulose or polystyrene. Also, the addition of excess RAP blocked toxin-mediated cell killing. These findings suggested that PE might utilize the LRP to gain entry into toxin-sensitive cells. Here we report on a strategy to select PE-resistant lines of CHO cells which display altered LRP phenotypes. An important part of this strategy is to screen PE-resistant clones for those that retain sensitivity to diphtheria toxin and to a fusion protein composed of anthrax toxin fused to the ADP-ribosylating domain of PE. At a minimum, this strategy should avoid the selection of EF-2 and acidification mutants. Analysis of seven independent lines revealed two classes of mutants: one class, with two members, had no detectable LRP while the second class, with five members, expressed LRP but at a reduced level. The LRP that was present in the second class was not processed efficiently into heavy and light chains. One line from each group was studied in greater detail. The 14-2-1 line had reduced amounts of cell-associated LRP, had no receptor light chain, did not internalize α2M-chymotrypsin complexes and was 10-fold resistant to PE. A second line, 13-5-1 was completely devoid of LRP, did not internalize α2M-chymotrypsin complexes and exhibited a 100-fold resistance to PE. Resistance to PE appeared to be due to receptor-specific defects, since these mutant lines showed no resistance to a PE chimeric toxin that was internalized via the transferrin receptor. Results confirmed that PE utilizes LRP to enter cells and that the use of several toxins in the selection and screening steps resulted in a high frequency of receptor-specific mutants.

Construction and activity of LRP ligand-toxin fusions.

Ligands that bind and enter cells via the heavy chain of LRP may or may not use the same pathway of endocytosis as PE. To investigate this, the DNA encoding the binding domain of PE was replaced with cDNAs encoding other LRP-binding ligands. These gene fusions were made between truncated versions of PE or diphtheria toxin (DT) and the LRP receptor associated protein (RAP). Likewise, a DT PAI-I fusion was made. Fusions proteins were expressed in *E. coli* and assayed for their ability to inhibit protein synthesis in several lines known to express LRP. Results indicated that the fusion proteins were less active than native PE.

Cleavage of *Pseudomonas* exotoxin and diphtheria toxin by a furin-like enzyme prepared from beef liver.

Pseudomonas exotoxin (PE) is cleaved within mammalian cells between Arg279 and Gly280 to generate an enzymatically active C-terminal fragment of 37 kD which translocates to the cytosol and ADP ribosylates elongation factor 2. A protease, with this toxin-cleaving activity, was prepared from beef liver and subsequently characterized. After achieving a 500-fold enrichment in several chromatographic steps, a soluble form of this protease was identified as a furin-like enzyme. It cleaved PE on the C-terminal side of the sequence of RQPR (amino acids 276-279) producing the same fragments as those generated within cells. Cleavage had a pH optimum of 5.0-5.5, 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). The beef protease cleaved PE with an apparent Km of 7 μM. A mutant form of PE, PEala281, was cleaved at the same site, with the same pH optimum, a similar Km (9 μM) but with a Vmax 150 times faster than was seen with the

native toxin. Mutational analysis of the amino acids located just before the site of cleavage, confirmed the importance of arginines at P1 and P4. It was also noted that the introduction of a dibasic pair at 278-279 did not increase toxicity or appreciably improve the rate of cleavage. Unnicked diphtheria toxin (DT) was also cleaved by the beef protease; cleavage was on the C-terminal side of the sequence RVRR (aa-190-193), was seen at pH values ranging from 5.5 to 8.5 and had an optimum at pH 8.0. Recombinant furin cleaved PE, PEala281 and DT with the same characteristics as the beef protease. In addition, Western blot analysis revealed that anti-furin antibodies reacted specifically with components in the beef protease preparation. Immunodepletion experiments showed that all toxin-cleavage activity could be removed from the beef protease using anti-furin antibodies. The relevance of furin-mediated cleavage was further assessed by adding nicked toxins to intact cells. Nicked PE and DT both killed cells at a faster rate than their unnicked counterparts.

Cleavage of chimeric toxins composed of TGFα fused with PE38.
To be active as a cytotoxic protein, PE must be proteolytically processed to produce a C-terminal fragment which translocates to the cytosol and inactivates EF-2. At pH 5.0-5.5, PE is cleaved between arginine 279 and glycine 280. No cleavage is seen at neutral pH. When either arginine 276 or arginine 279 of PE are changed to glycine there is no cleavage and no toxicity. It is not known if chimeric toxins, composed of a targeting ligand fused to domains II and III of PE, also require a similar form of proteolytic processing. To study this, we have produced TGFαPE38 and assayed its sensitivity for cleavage by furin, the protease that is believed to cleave PE. Furin cleaves TGFαPE38 at low pH but not at neutral pH and although the digestion products have not been sequenced, cleavage appears to occur at the same site as in native PE. For further investigation, two mutant forms of TGFαPE38, TGFαPE38PEgly276 and TGFαPE38gly279, were constructed. Mutant proteins were expressed in *E coli* and then characterized for their cytotoxic activity and their sensitivity to furin-mediated cleavage. Depending on the cell line the mutant forms were 10-100-fold less active than the wild type chimeric toxin and neither was a substrate for furin mediated cleavage. The latter result was expected since furin requires a basic amino acid at both the P1 and P4 positions relative to the site of cleavage. Results suggest that chimeric toxins, composed of a binding ligand fused to domains II and III of PE, require the same proteolytic processing as native PE.

Targeting toxins to myeloid leukemia cells.
Hematologic malignancies should be readily accessible to soluble protein toxins. Monoclonal antibodies were obtained that bound selectively to human myeloid leukemia cells. Two of these, termed 251 and 7AG11, were coupled to lysPE38 by a thioether bond. The resulting immunotoxins were tested for cytotoxic activity on several leukemic cell lines. The 251-lysPE38 was most active on K562 cells with ID50s ranging from 20-200 ng/ml.

Publications:

Zdanovsky AG, Chiron M, Pastan I, FitzGerald DJ.: Mechanism of action of *Pseudomonas* exotoxin - identification of a rate-limiting step. J Biol Chem 1993;268:21791-9.

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

PROJECT NUMBER
Z01 CB 08758-03 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies of transcription mechanisms and structure-function relationships of *E. coli* RNA polymerase

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

PI:	Ding Jun Jin	Senior Staff Fellow	LMB, NCI
Other:	Ming Xu	Special Volunteer	LMB, NCI
	Matthew A. Fisher	IRTA Fellow	LMB, NCI
	Yan Ning Zhou	IRTA Fellow	LMB, NCI

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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B

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

The goals of this project are as follows: (1) Analysis of transcription mechanism(s) by comparing the differences between wild-type and mutant RNA polymerases (RNAPs) in the processes. (2) Determination of structure-function relationships of *E. coli* RNAP by isolation and characterization of mutant RNAPs that have altered particular functions in transcription.

Study of promoter clearance during transcription initiation has been continued. Analyzing the parameters and the site(s) in RNAP that affect nonproductive synthesis of stuttered products at *galP2* are focused upon. Stuttering synthesis at *galP2* is sensitive to changes in UTP concentration and is repressed by cAMP-CRP *in vitro*. The *in vivo* expression of *galP2* also appears to be sensitive to changes in internal UTP pools, indicating that modulation of promoter clearance at *galP2* is biologically significant. An RNAP mutant, which has deleted the Ala532 residue in the β subunit, has dramatically reduced its stuttering synthesis at *galP2*, because it has increased its efficiency in incorporating the CTP at the fifth position of the *galP2* transcript indicating that the rate of synthesis of that nucleotide in the initially transcribed sequence is a critical or rate-limiting step for promoter clearance. A check point model for promoter clearance at *galP2* is proposed.

The interaction between RNAP and *pyrBI*, a promoter which shares some characteristics with known stringently controlled promoters is analyzed. Transcription at *pyrBI* is activated by supercoiled DNA at high salt concentrations, probably due to an enhanced stability of the open complex formed. Isolating and characterizing the mutant RNAPs that have altered transcription initiation at *pyrBI* are planned.

The nature of drug(s) resistance of RNAP in *E. coli* and other pathogenic bacteria is determined. Eighteen different Rifr mutations from *Staphylococcus aureus* are sequenced and found to have the same or similar amino acid residues changes as that in *E. coli*, indicating that the rif-region is highly conserved in eubacteria. The sensitivity of Rifr RNAPs to several antibiotics is determined and found to have a different degree of cross-resistance. The cross-resistance of different drugs is correlated with overlapping binding sites in RNAP.

Major Findings:

I. Studying the biochemical mechanism(s) of promoter clearance and the effects of the mutant RNA polymerases (RNAPs) conferring rifampicin-resistance (Rif^r) on this process. Transcription initiation by *Escherichia coli* RNAP consists of multiple steps as follows: (1) RNAP recognizes a promoter and binds specifically to form a "closed complex"; (2) after one or more intermediate steps, the closed complex isomerizes into an "open complex" in which about 12 nucleotides of DNA are melted; (3) with the addition of nucleotides, an initial transcribing complex is formed; (4) the initial transcribing complex clears the promoter and enters an elongation mode. The effects of the first two steps in transcription initiation have been studied extensively. However, our knowledge about the effects of the later steps on transcription initiation is limited. During promoter clearance (step 4 above), a transition step between transcription initiation and elongation, RNAP makes two kinds of nonproductive initiation transcripts: aborted and stuttered products. Previously, Jin showed that one Rif^r RNAP mutant, RpoB3401, has overproduced abortive initiation products and reduced productive transcription at the *pyrBI* promoter, because it has reduced affinity for UTP indicating the importance of Km of RNAP for nucleotide(s) in controlling the abortive initiation. Recently, he has found that stuttering synthesis is a means to regulate transcription at the *galP2* promoter of *Escherichia coli*. Jin has tried to determine the parameters and the site(s) in RNAP that affect nonproductive synthesis of stuttered products at *galP2* and the effects of stuttering synthesis on promoter clearance and productive synthesis at the *galP2* promoter.

A. RNAP makes nonproductive stuttering synthesis at the *E. coli galP2* promoter

1. *In vitro* transcription shows that RNAP produces stuttering initiation products at the *galP2* but not *galP1* promoter. The stuttered products are pppAUUUUn (n can be 1 to more than 20) and are pseudo-templated from *galP2* that has the initially transcribed sequences pppAUUUC.

2. The stuttering synthesis from *galP2* is sensitive to changes in UTP concentration and is enhanced at higher UTP concentrations. The stuttering synthesis from the *galP2* promoter is inhibited by the cAMP-CRP complex.

3. The *in vivo* expression of *galP2* is also sensitive to the changes in the internal UTP pools. The correlation between the *in vitro* and the *in vivo* results indicates that the stuttering synthesis at *galP2* has biological significance.

B. RpoB3449, a mutant Rif^r RNAP having an Ala532 deletion in the β subunit, has dramatically reduced stuttering synthesis from the *galP2* promoter, indicating that part of the rif-region is also important for stuttering synthesis. The mechanism by which RpoB3449 has altered stuttering synthesis is analyzed, and the effects of the mutant RNAP on promoter clearance and on the production of the full length *galP2* transcripts are determined.

1. RpoB3449 has "normal" stuttering synthesis at the *pyrBI* promoter but has reduced stuttering synthesis at *galP2*, indicating that the mutant RNAP has altered initiation specifically at *galP2*.

2. The mutant RNAP has increased the rate of incorporation of CTP at the fifth position of the *galP2* transcript resulting in reduced stuttering synthesis. When the concentration of

CTP is reduced enough, RpoB3449 is able to make significant amounts of stuttered products at *galP2*, and conversely, when the concentration of CTP is high enough, wild-type RNAP simulates the phenotype of the mutant RNAP in stuttering synthesis at *galP2*. These results indicate that the rate of incorporation of CTP at the fifth position (after a stretch of uridine residues) of the *galP2* transcript is a critical or rate-limiting step in promoter clearance. A check point model for promoter clearance at *galP2* is proposed to account for the stuttering synthesis at the promoter.

3. The nature of the initial transcribing complexes that have produced a stuttered product at *galP2* is determined. Apparently, during stuttering synthesis, wild-type RNAP makes a multiple round of initiation without dissociation from the promoter before entering the elongation mode and completing a productive initiation cycle. Therefore, promoter clearance is a rate-limiting step for productive synthesis of the full length *galP2* transcript. RpoB3449 has a faster rate of productive synthesis of *galP2* due to its faster rate of promoter clearance compared to wild-type RNAP. A direct link between promoter clearance and productive initiation is demonstrated.

C. Jin has found that nonproductive stuttering synthesis also occurs at the *cya* and *carA*(*pyrA*) genes, indicating that the expression of a set of *E. coli* genes is also subject to promoter control and is sensitive to changes of nucleotides pools inside cells. Apparently, RNAP is able to respond to its substrates and, in turn, to modulate the transcription activity.

II. Studying the interaction between RNAP and the *pyrBI* promoter. The *pyrBI* promoter shares some characteristics with the known stringently controlled promoters, and it serves as a model system for studying the interaction between RNAP and this class of promoters biochemically and genetically.

A. Transcription at the *pyrBI* promoter is activated by supercoiled DNA.

1. Jin has found that transcription of the *pyrBI* promoter at high salt concentration is enhanced by having supercoiled DNA template. Transcription from a supercoiled DNA template is more resistant to heparin challenge than from a linear DNA template. As measured by transcription activity, the half life of the transcription complex of a supercoiled DNA template is about >100-fold longer than that of a linear DNA template.

2. Fisher has determined the stability of open complexes formed at the *pyrBI* promoter by fluorescence detected abortive initiation assay. The half life for RNAP dissociating from the open complex is 2 min and 70 min for linear and supercoiled DNA template, respectively, indicating that supercoiled DNA stabilizes the open complex. The bimolecular association rate constant calculated from nitrocellulose filter binding experiments using linear DNA is approximately 10^8 M^{-1} sec^{-1}. This value is in the range expected for a three dimensional diffusion limited process, supercoiling is unlikely to enhance this.

III. Study the interactions between core RNAP and different σ factors. The conserved regions 2 and 3 of σ factor(s) were identified in the binding to core RNAP. However, the regions in core RNAP that are involved in the binding to σ factor(s) are unknown. Zhou is initiating this study by analyzing some known mutant core RNAPs, which appeared to have altered interactions with σ factor(s) phenotypically, to determine whether they have altered the binding to σ factor(s). Currently, in collaboration with Richard Calendar at UC-Berkeley, Zhou has being analyzing some *rpoH* mutants that might have altered the binding to core RNAP. Her preliminary results not only confirmed that the conserved regions 2 and 3 of σ^{32} are involved in the core binding but also suggest that region 4 is important for the core binding. Zhou is planning to isolate intergenic suppressers in core RNAP that will correct the defects of these mutant σ^{32} in binding to core RNAP.

IV. Identification of rif-region in pathogenic bacteria and study of cross-resistance of Rif^r RNAP to other antibiotics. With funding from a pharmaceutical company, this part of the work is an attempt to overcome the clinical problems of emerging Rif^r mutants of infectious microorganisms.

A. To understand the nature of the Rif^r mutations in pathogenic bacteria *Staphylococcus aureus,* Xu has sequenced 18 clinically different isolates of Rif^r mutation from the bacteria. Only four amino acid residues are affected, and these affected residues are the same or conserved ones as those in *E. coli*, indicating that the rif-region is highly conserved in eubacteria.

B. The target of several antibiotics including rifampicin is RNAP in bacteria. Xu has determined the sensitivity of Rif^r RNAPs to these antibiotics and found different degrees of cross-resistance. Two rifampicin derivatives, rifabutin and rifapentane, are found to be completely cross-resistance with rifampicin. A new class of antibiotic, sorangicin A which is unrelated to rifampicin, is found to be partially cross-resistant to rifampicin. About half of the purified Rif^r RNAPs were resistant to a different degree to sorangicin A; whereas, 100% of sorangicin A resistant mutants cells have acquired Rif^r phenotype. These results suggest that the binding sites for rifampicin and sorangicin partially overlap. Indeed, Xu found that sorangicin A inhibits the binding of rifampicin to RNAP just as effectively as the two rifampicin derivatives, further suggesting that the binding sites for rifampicin and sorangicin A are shared. The known 3-D structures of rifampicin and sorangicin A molecules will be analyzed to postulate the potential interaction sites between antibiotics and RNAP. The information could be potentially useful for designing new antibiotics that will inhibit Rif^r RNAPs.

V. Collaborating studies on the effects of mutant RNAPs on different aspects in transcription. Previously, by studying the effects of Rif^r RNAPs that have altered elongation rates on termination, Jin found that there is a kinetic component in the control of transcription termination at the rho-dependent terminator. Since these mutant Rif^r RNAPs that have altered termination at rho-dependent terminators also have affected termination at the intrinsic terminators, it suggests that the kinetic control is also important for termination at the latter terminators. To test this model, Jin is collaborating with Dr. Jeffrey Roberts' group at Cornell University to study the

effects of these mutant Rif^r RNAPs on termination at an intrinsic terminator. The results are supporting the model. In collaboration with Dr. Robert Weisberg's group at NICHHD, NIH, Jin has purified a RpoC mutant RNAP that is defective in antitermination of early transcription of phage HK022 and performed *in vitro* transcription assays to confirm its defect biochemically. Also, Jin collaborated with Dr. Timothy Donohue's group at University of Wisconsin-Madison to purify RNAP from *Rhodobacter sphaeroides.* Multiple forms of holoenzymes with different sigma factors are identified.

Publications:

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

PROJECT NUMBER
Z01 CB-08759-03 LMB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

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

PI:	B. K. Lee	Chief, Molecular Modeling Section	LMB, NCI
Others:	N. Kurochkina	Visiting Fellow	LMB, NCI
	S.H. Jung	Visiting Fellow	LMB, NCI
	B. Madan	Visiting Fellow	LMB, NCI
	J. de la Cruz	Visiting Fellow	LMB, NCI
	Y. Sergeev	Exchange Scientist/Scholar	LMB, NCI
	J. Cammisa	Computer Specialist	LMB, NCI

COOPERATING UNITS (if any)
Laboratory of Molecular Biology

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Modeling Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.3	5.0	1.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The Molecular Modeling Section uses and develops theoretical tools with which to study the forces that govern the structure and interaction of globular protein molecules, to study and predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties. In the past year, we: (1) clarified the relation between the enthalpy-entropy compensation phenomenon and hydrophobicity, (2) found additional evidence that size of the water molecules is an important factor in determining the hydrophobicity, (3) obtained a 3 Å structure for the crambin molecule by guiding the folding process, (4) devised and tested a simplified but rapid way of estimating the hydrophobic energy to be used in protein folding studies, and (5) devised a structural alignment scheme tailored to the α/β barrel structures and found evidence of sequence shuffling in some members of this family of proteins. We also studied hybrid molecules of the *Pseudomonas* exotoxin (PE) with different antibody Fv's, with interleukin 4, and with TGFα. We made a number of recommendations to improve various properties of these molecules by site-directed mutagenesis, some of which have been tested and found to have the desired property.

Major Findings:

A. Software maintenance (with 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. We incorporated numerous improvements in these programs in the past year. Particularly notable is the incorporation in GEMM of a subroutine that controls molecular movements using the robotic arms technology. This feature makes possible a totally new type of control in the manipulation of molecules. The subroutine was written by the Summer students R. Lim and B. Doran with the assistance of J. Cammisa. The routine, as it stands now, is rather rudimentary and must be improved before it can be of general and routine use.

As in previous years, we assisted a number of people in the Section, in the Laboratory, and even outside the Laboratory, in the use of these programs and in some cases helped them directly by producing color pictures of the molecules of their interest. We have also continued to distribute the program to people all over the world on request.

B. Hydrophobic effect (with B. 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 has traditionally been considered to be due to the formation, in the aqueous solution, of an "iceberg-like" structure around the solute. In an effort to prove or disprove this hypothesis, we started to make simulation calculations about one and a half years ago using a realistic water model and with another liquid which is identical to this water model except that the hydrogen bonds were turned off. (This operation cannot be done in nature, but is quite easy to do in a computer.) We finished this project during last year. We showed that the hydrophobic effect was stronger in the liquid without the hydrogen bonds and that it is sensitive to the size of the molecule. The study provides a strong support for the notion, advanced several years earlier, that the hydrophobic effect arises from the small size of water molecules. The manuscript describing this work is in press.

The estimation of the magnitude of the hydrophobic effect depends on the standard state chosen to represent the experimental thermodynamic quantities. Once in a while, some workers propose a substantially large estimate for the effect by using unconventional standard states. The most recent episode of this type occurred in 1991 when Sharp, Honig and their coworkers suggested that the magnitude of the effect should be nearly twice as large as that commonly accepted. This suggestion remains controversial to this date. In the past year, we clarified the nature of this latest proposal and worked out the proper standard state to use. The manuscript describing this work is also in press.

C. Modeling crambin to 3Å accuracy by guided folding (with N. Kurochkina)

We obtained a structure of crambin which deviated from the x-ray structure by 3.0 Å by manually guiding the folding process. This was achieved by using a multi-step Monte Carlo procedure and assuming that the disulfide bonds are known. Briefly the procedure is as follows:

The energy function used was the sum of the Miyazawa-Jernigan contact potential and the hydrogen bond energy represented as a Coulomb sum. The relative weight between the two energy terms was determined by running the folding program to minimize the rms deviation from the x-ray structure.

The actual search was done using this potential and restricting the possible dihedral angles to 8 (Lim et al.) or 6 (Wodak et al.) states per residue in order to reduce the search space. In the first step, only those structures that form the inner-most disulfide bond best were selected among a set of low energy structures. The structure up to the inner-most disulfide bond was then frozen and the process repeated to find the structues that form the next disulfide bond best. The procedure was then repeated a third time for the last disulfide bond.

Although this is not a prediction, since the procedure uses the knowledge of the structure, it does show that an acceptably accurate structure can be obtained if the folding process is correctly guided. This work was presented at the NATO workshop on Statistical Mechanics, Protein Structure, and Protein-substrate Interactions, the proceedings of which will be published.

D. Simplified hydrophobic potential (with N. Kurochkina)

Our previous thermodynamic study on protein stability and our experience with the Monte Carlo folding program strongly indicate that the hydrophobic and the polar interactions are about equally important in protein folding. The main component of the polar interaction is the hydrogen bonding, which can be handled adequately by means of simple Coulomb sum. The hydrophobic force, on the other hand, is obtained from the change in the accessible area. Whereas the Coulomb sum is easy to compute, the surface area calculation is prohibitively expensive. For this reason, we relied in the past on the residue-level empirical potentials to assess the hydrophobic force.

In the past year, we sought and found a way to compute the atom-level hydrophobic energy fast. It is based on pair-wise sum and takes as much time as the Coulomb sum. The calculated value is only an approximation to the full hydrophobic energy but, according to a number of tests we made, the approximation appears to be good enough to be used in protein folding applications. For exmple, we found that the new potential can correctly distinguish the native structure of the helical protein ROP from an ensemble of structures generated by the protein folding program. A manuscript describing this new way of approximating the hydrophobic potential is now in preparation.

E. Structural pattern recognition for the α/β barrel motif (with Y. Sergeev)

The α/β 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 differrent functions and essentially no sequence homology among them. Although the α/β 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 α/β barrel is a challenging case for automatic structural pattern recognition, for the structure-based sequence alignment procedures, and for recognition by threading algorithms.

Last year, we reported on devising a new scheme, specific to the α/β barrels, by which the structural pattern can be characterized and unambiguously aligned. When we aligned some 10 α/β barrel structures following this scheme, we found that about half of the proteins could be aligned normally, but three or four other proteins were best aligned after permuting the sequence in blocks. This means that gene for some members of this family of proteins must have been shuffled some time in their evolutionary past. Since most of the sequence and sequence-structure alignment procedures do not allow the possibility of such sequence shuffling, it is not surprising that they do not work well with these proteins. A manuscript reporting this finding has been submitted.

F. Specific molecular modeling (with S.-H. Jung)

Among numerous modeling projects that we carried out in the past year, mostly in collaboration with the immunotoxin group, the following are probably the most noteworthy.

(a) Following the suggestion by Novotny and his coworkers, we identified possible epitopes (or antigenic residues) in domains II, Ib and III of PE toxin by calculating the accessible surface area of each residue with 10 A probe. The calculated surface area were then compared with the experimental data for antigenic residues from the peptide scanning data and from competition and neutralization assays using single residue mutants and different segments of PE toxin. There were some significant, although not perfect, correlation. The work has been submitted for publication.

(b) On the basis of the pattern of change in activity of the interleukin 4 (IL4) - toxin conjugates, a suggestion was made to cyclically permute the IL4, i.e. join the carboxy and amino terminals of the molecule and, instead, break the molecule in the middle. The suggested molecule has been synthesized by Dr. R. Kreitman in the immunotoxin group and found to be fully active. The manuscript describing this work is in preparation.

(c) In an effort to understand the role of the interaction between the V_L and V_H domains in the stability and the antigen binding affinity of the antibody Fv, we have studied the V_H-V_L domain orientation in different antibodies and its change upon antigen binding. We found that antibodies of known structure come in two classes, one in which the antigen binding produces relatively minor changes in the relative orientation between the two domains and the other in which the antigen binding apparently induces relatively large and complex changes. We are currently investigating the features that delimit these two different types.

(d) On the basis of the structural comparison between PE and the diphtheria toxin, suggestions have been made on how to modify domain III of PE to reduce its size. Size reduction is clinically important for immunotoxins since small molecules will leave capillaries faster, thereby reducing the chance for non-specifc toxic effect on the cells lining the endothelial cells, and because they will better penetrate into the interior of solid tumors. The suggested molecules have yet to be made and tested.

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SUMMARY
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS
NATIONAL CANCER INSTITUTE
1994

ABSTRACT

The Laboratory of Pathology integrates anatomic pathology service and training with programs in molecular pathology research. The Laboratory is responsible for all the anatomic pathology diagnostic services for the NIH Clinical Center. A fully accredited 4-year residency program is provided for 9 residents and 3 fellows. Clinical Center patient diagnostic specimens and autopsies, extramural consult cases sent in from around the world, and pathology rotations at local institutions, all provide the teaching material. A spectrum of basic and applied research activities constitute a rich choice of opportunities for residents to conduct investigative work.

The Laboratory is subdivided into a series of sections and units. Each subgroup is oriented around one of four areas of focus: 1) basic research, 2) core laboratory support, 3) translational research and training, and 4) clinical service. This organizational structure encourages the full extension of basic research discoveries originating within the Laboratory into novel diagnostic markers and treatments. The Laboratory strives to apply its special combination of expertise and human tissue material to originate new methods in molecular pathology and apply them to fundamental disease questions.

Molecular Immune Activation Section (Dr. Kathleen Kelly, Chief)
Office of the Chief (Dr. Susan Mackem)
Gene Regulation Section (Dr. David L. Levens, Chief)
Biochemical Pathology Section (Dr. David D. Roberts, Chief)
Extracellular Matrix Pathology Section (Dr. W. G. Stetler-Stevenson, Chief)
Women's Cancers Section (Dr. Patricia S. Steeg, Chief)
Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
  Signal Transduction and Prevention Unit (Dr. Elise C. Kohn, Chief)
Molecular Pathology Section (Dr. Mark E. Sobel, Chief)
Surgical Pathology Section (Dr. Maria J. Merino, Chief)
Cytopathology Section (Dr. Diane Solomon, Chief)
Postmortem Pathology Section (Dr. David E. Kleiner, Chief)
Ultrastructural Pathology Section (Dr. Maria Tsokos, Chief)
Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
  Specialized Diagnostics Unit (Dr. Mark Raffeld, Chief)
  Flow Cytometry Unit (Dr. Maryalice Stetler-Stevenson, Chief)

Molecular Immune Activation Section

The goal of the Molecular Immune Activation Section is to identify proteins that regulate the activation process in lymphoid cells and to characterize the biochemical basis of their activity. As a means of identifying novel proteins potentially involved in the activation process, Dr. Kathleen Kelly isolated several activation-specific cDNA clones from mitogen-stimulated human peripheral blood T cells. Primary sequence analyses have been used to identify potentially interesting functional classes of proteins. In particular, Dr. Kelly's lab has concentrated on a small number of novel proteins with potential signal transducing functions as such proteins might be expected to play regulatory roles in the control of proliferation and differentiation that occurs during lymphocyte activation.

One such induced protein identified and characterized by Dr. Kelly's lab is PAC1, a hematopoietic cell-specific, nuclear protein phosphatase with dual specificity for threonine and tyrosine. Dr. Kelly's lab has used several approaches to demonstrate that PAC1 is a physiologically-relevant MAP kinase phosphatase. MAP kinase activation is a central component of signal transduction pathways initiated by several growth and differentiation factors and oncogenes. The initial activation of MAP kinase is reversed by PAC1 assuring the transient nature of this early signal transduction pathway.

Another protein being studied is GEM, a 35 kD GTP-binding protein that appears to be distantly related to the ras family. GEM expression is transient and is correlated with activation in several cell types. GEM is located on the inner face of the plasma membrane suggesting that it may play a role in signal transduction. Constitutive overexpression of GEM leads to cell death. The mechanism of such death is currently under investigation and may give a suggestion as to the physiological role of GEM.

A third signalling molecule being studied is 276. 276 codes for a precursor protein that is processed to a cell surface protein and a seven pass integral membrane protein that belongs to that class of receptors that couples signal transduction through trimeric G proteins. 276-encoded proteins are broadly expressed on hematopoietic cells. The extracellular domain contains several EGF repeats (motifs often seen in receptors or their ligands) and an RGD sequence that suggests a potential interaction with a certain class of adhesion molecules. The extracellular domain is associated with the cell surface although it is not covalently bound to the integral membrane protein. It is reasonable to suggest, however, that the extracellular domain and the integral membrane components may have coupled functions. The most immediate goal of this project is to identify the ligand for the extracellular domain. It is possible to express the extracellular domain as a soluble product making various biochemical approaches feasible.

Office of the Chief

Dr. Susan Mackem's laboratory is interested in elucidating the mechanisms at the molecular level, by which pattern formation is regulated during embryonic development. Limb morphogenesis in the chick is being studied as a model system for pattern formation that is readily amenable to various experimental manipulations. Induction of the primary embryonic axis during gastrulation is also being studied, as there appears to be an interesting concordance of developmental control genes that regulate both of these processes. Using pcr-based strategies to identify new members of the homeobox gene family expressed specifically in developing limb buds, two novel non-*Antennapedia* homeobox genes have been identified, which are currently a major focus of investigation in the laboratory. These genes have been extensively characterized with respect to their temporospatial expression domains in the embryo by *in situ* hybridization of both histologically sectioned, and of "whole mount" embryos. A combination of several biochemical and molecular genetic approaches are being employed to analyze the functions of these genes, including transgenic approaches in the mouse.

One of these homeobox genes, *Ghox d-12* (formerly *Ghox 4.7*), belongs to the *Hox d* cluster, and along with several other *Abd B*-like homeobox genes located in this cluster, is expressed in a highly restricted posterior domain of the early limb bud. Overlapping, nested posterior expression domains of *Ghox d-12* and several other *Hox d* genes in the limb bud has suggested a role for these genes in regulating pattern along the anterior-posterior axis of the limb bud. The laboratory has generated preliminary results in transgenic mice that support this hypothesis. Ectopic expression of *Ghox d-12* in the anterior region of the developing limb bud results in a transformation of anterior limb skeletal elements to a more posterior-type of identity (eg. digit one is converted to digit two, and digit two to digit three).

The other gene, *Gnot1* (formerly *L5*), has an unusual homeodomain with some homology to *drosophila* EMS, and is most closely related to a recently described *xenopus* gene, *Xnot*. *Gnot1* is expressed both during gastrulation, and later selectively in the limb bud. The expression is highly restricted along the proximo-distal axis of the limb, and is dynamic, correlating with the spatial zones destined to give rise to the wrist/ankle region, which become progressively determined and proximally displaced as outgrowth of the limb proceeds. Microsurgical manipulations that alter the developmental program of the limb to produce duplicated skeletal patterns also result in a duplication of spatial expression of *Gnot1* in the limb bud, indicating that the expression of this gene is correlated with a particular positional identity. Hence, *Gnot1* may play a role in establishing pattern along the proximodistal limb axis.

During early development, *Gnot1* is initially expressed in the hypoblast, which is thought to determine the orientation of the embryonic axis in the chick. At the onset of gastrulation, expression becomes restricted to Hensen's node, which is thought to be the equivalent of Spemann's organizer in chick. As gastrulation proceeds and the notochord arises anteriorly from Hensen's node, *Gnot1* is also expressed in a dynamic wave along the notochord

that correlates spatially with the level of ongoing segmentation of the adjacent paraxial mesoderm into somites down the anterior-posterior axis of the embryo. These transient early expression domains suggest that *Gnot1* plays a major role in regulating the formation of the primary embryonic axis during gastrulation and in the progression of somitogenesis via inductive signals from the notochord.

Long-term experiments to determine the function of *Ghox d-12* and *Gnot1* are currently underway, and include altering the expression of these genes using transgenic technology in mice and avian retroviral expression vectors in chick embryos, as well as antisense oligonucleotide inhibition experiments in embryo cultures, to look at loss-of-function and gain-of-function effects. Biochemical approaches, including immunoselection of endogenous, *in vivo* protein-DNA complexes, are also being employed to identify individual, immediate downstream "target" genes that are regulated by *Ghox d-12* and *Gnot1* during morphogenesis. These downstream genes may be expected to include the mediators, as well as regulators, of these developmental processes.

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).

1) 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 differentiation of promonomyelocytic 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. 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 cDNA encoding the FUSE binding protein (FBP). The FBP possesses a novel structure including a new DNA binding motif. Surprisingly, recombinant FBP binds most avidly to a single strand of FUSE. Expression of FBP itself is regulated, being shut off during differentiation. Transfection of vectors expressing FBP stimulates c-myc promoter mediated expression and deletion of the FUSE element diminishes this stimulation. Because of the unusual single strand specificity of FBP, *in vivo* methods were sought to determine if the augmentation of c-myc promoter

mediated expression might be related to the conformation of the FUSE. Modification of DNA *in vivo*, by treatment of cells with $KMnO_4$ followed by piperidine treatment and ligation mediated PCR demonstrates that FUSE has single strand properties *in vivo*. Screening for genes related to FBP has revealed the existence of other highly related proteins indicating that FBP may be the prototype for a new family of gene regulatory proteins.

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 a well characterized RNA binding motif and has no homology to other hnRNP proteins. Surprisingly, hnRNP protein K clearly possesses the same DNA binding motif present in FBP.

Transfection of vectors expressing hnRNP protein K mRNA or anti-sense hnRNP protein K RNA demonstrate that hnRNP protein K stimulates CT-element mediated expression whereas decreased levels of this protein diminish expression. Cleavage of the transfected reporter plasmids with potassium permanganate indicate that hnRNP protein K may facilitate the local denaturation of the CT-element. A second factor binds specifically to the complementary GA strand. This protein is a previously undescribed variant of a known protein, CNBP, and apparently arises from an altered splice. CNBP can also bind the CT element. Surprisingly, the two forms differ in their DNA binding properties. A variety of biochemical studies, especially those employing transcription, *in vitro*, indicate that hnRNP protein K and CNBP interact with each other and with components of the basal transcription machinery. The well-characterized transcription factor SP1 can also stimulate transcription through the CT-element under some conditions and may participate in the formation of a larger complex at this site.

Further studies of the FUSE, the CT-element, their DNA conformations and their associated proteins may help to elucidate the rules governing c-myc expression.

Previously, a cis-element was identified in intron I 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 that the myc-intron binding protein is a member of the RFX (regulatory factor HLA DR X box)/MDBP-methylation dependent binding protein) family of DNA binding proteins. However, the peptide sequences indicate that related members of

this family may exist and bind to the intron site. Cloning of other RFX members is currently in progress. Concurrent transfection studies comparing c-myc reporter genes with wild-type or deleted RFX-intron binding sites reveals that this sequence is a positive cis-element in Raji cells, a Burkitt lymphoma cell line. Because this site is mutated in most Burkitt lymphomas, including Raji, loss of a positive element was unexpected as c-myc over-expression is usually thought to be associated with tumorigenesis. The potential linkage between c-myc and MHC class II expression revealed by binding of RFX may suggest an interesting coupling between antigen presentation and clonal selection governing the growth and death of cells in germinal centers; abberations in this process may contribute to the pathogenesis of Burkitt lymphoma progress.

2) The mechanism of transactivation by *junD*/AP-1 complexes in T-cell activation

Prior studies in the section have identified an AP-1 consensus sequence within the 22 bp principal enhancer element of the gibbon ape leukemia virus, (a member of the family of type C retroviruses associated with several distinct hematopoietic malignancies). Subsequent studies have shown that the gibbon T-cell lymphoma cell line, MLA 144 contains factors that strongly transactivated this AP-1 enhancer element (GALV-AP1) *in vivo*.

Efforts to isolate these factors have led to the identification of a novel multicomponent transcription factor complex (T-AP1) that pre-exists in resting T-cells. T-AP1 is induced during T-cell activation by the addition of phorbol esters. This induction occurs in the absence of protein synthesis, and therefore, suggests that T-AP1 may play a significant role in the earliest stages of T-cell activation through rapid modulation of its function by post-translation modification.

The T-AP1 complex has been purified over 9000 fold from MLA 144 cells and has been found to be formed from multiple components. One component is comprised of *junD* 43 kD and 38 kD polypeptides. A second component, identified by its partial dissociation from T-AP1 during purification, has been purified. This component, termed "activator" is comprised of multiple low molecular weight polypeptides between 27-23 kD with two major polypeptides of 24 and 23 kD. Activator is able to stimulate purified *junD* DNA-binding over 100 fold, and can increase AP-1 enhancer directed transcription *in vitro*. The function of the activator is highly specific for *junD* since it inhibits rather than stimulates c-*jun* DNA binding activity *in vitro*. The DNA-binding activity of the T-AP1 complex is blocked by acid phosphatase, implicating phosphorylation as a necessary post-translational modification for function. This observation is further underscored by the reduced responsiveness of recombinant *junD* to purified activator.

JunD and the activator constitute the essential elements of the T-AP1 complex since gel purified and renatured *junD* 43 or 38 kD polypeptides in combination with either of the renatured activator polypeptides (27-23 kD) can reconstitute the TAP-1 complex. Numerous properties of purified T-AP1 distinguish it from *junD*. T-AP1 binds DNA with a 10 fold higher affinity, has

a 90 fold longer half-life, makes twice as many contacts with the flanking DNA sequences contained within the GALV-AP1 site, and shows different relative DNA specificity by comparison to recombinant *junD*.

Interestingly, the activator has also been found to modulate the binding of other AP-1 containing complexes involved in T-cell activation. One of these complexes, NF-AT (nuclear factor of activated T-cells) plays a major role in the induction and T-cell restricted expression of IL-2 during T-cell activation. NF-AT has been found to be composed of a cyclosporin A sensitive component variably complexed with different AP-1 family members, including *junD*, *c-jun*, *c-fos*, and *FosB*. The activator is able to stimulate over 50 fold the binding of NF-AT to its cognate binding site in the IL-2 promoter and also stimulates transcription from a NF-AT enhancer driven promoter *in vitro*.

A second important enhancer element in the IL-2 promoter is NF-IL2A. This site has been shown to be transactivated by the oct/OAP (octamer/octamer associated protein) complex which has recently been found to contain *junD* as a major constituent. Preliminary experiments indicate that activator is able to stimulate the DNA binding of this complex at least 5-10 fold.

It appears that the activator may have a general function as an adaptor protein that augments and stabilizes interactions between *junD/AP1* containing complex and specific enhancer elements in T-cells. The IL-2 promoter also contains functional AP-1 sites. Therefore, as a positive modulator of 3 distinct *junD/AP1* containing complexes that are highly active at 3 distinct enhancers within the IL-2 promoter, the activator appears poised to assume a major role in T-cell activation.

Current efforts are directed at generating sufficient purified activator for peptide sequencing so that sequences suitable for obtaining a full-length cDNA encoding the activator may be derived. Preliminary evaluation of peptide sequence obtained thus far suggests that the activator is a unique protein.

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 that mediate 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 *Candida albicans* 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. Specific amino acid sequences in thrombospondin that express these activities have been identified, and the cell receptors recognizing these regions of the molecule 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 1 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 cell proliferation. A peptide from the second type 1 repeat binds specifically to fibronectin and inhibits fibronectin-mediated interactions of cells with type 1 collagen.

Several approaches were 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. Recombinant fragments of thrombospondin containing these sequences bound to heparin with a similar specificity to that of the native protein. In contrast, binding of peptides from the type 1 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.

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. The association of increased expression with decreased tumor growth and metastatic potential could result from direct effects on the tumor cells or from inhibition of neovascularization of the tumors. Thrombospondin inhibited endothelial cell motility and proliferation in response to basic fibroblast growth factor (bFGF). These activities were mimicked by the recombinant amino-terminal domain and by the type 1 repeat peptides. These molecules also competed with bFGF for binding to endothelial cells, demonstrating that they act, at least in part, by inhibiting bFGF binding to its low affinity proteoglycan receptor. These results suggest that the synthetic peptides from thrombospondin may be a useful agent to regulate angiogenesis. Preliminary studies using a conjugate of the thrombospondin peptides demonstrated inhibition of angiogenesis and tumor growth in two animal models.

Extracellular Matrix Pathology Section

Extracellular matrix (ECM) degradation is a key step in many physiologic as well as pathologic processes. Normal physiologic events such as wound healing and trophoblast implantation require carefully controlled synthesis and removal of ECM components. These normal processes are strictly regulated in a spatial and temporal fashion, and are usually of limited extent. Physiologic matrix remodeling results in a functionally intact matrix and preservation of tissue boundaries. ECM turnover associated with many pathologic processes can take place in an aberrant and destructive fashion resulting in a dysfunctional matrix and loss of normal tissue boundaries. Such extensive destruction can be seen in inflammatory collagen vascular diseases such as rheumatoid arthritis. Matrix turnover associated with other pathologic conditions, such as angiogenesis as well as the invasion and metastasis of neoplastic cells, may be more limited in original extent but are dysregulated in that they are unresponsive to physiologic arrest signals. We are studying the regulation of ECM turnover in both pathologic and physiologic conditions with the aim of developing new strategies for limiting tissue destruction and the development of disease states. This understanding may allow selective disruption of extracellular matrix destruction that is associated with pathologic conditions, restoring normal matrix organization and preserving matrix function.

The Extracellular Matrix Pathology Section is studying the development of the invasive phenotype in normal and neoplastic cells. Historically, the process of cellular invasion has been divided into three steps: cell attachment to the matrix, lysis of matrix components and migration of the invading cell through the matrix defect. Specific gene products have been identified for critical functions in each of these three steps. With respect to matrix degradation we have focused on the role of the matrix metalloproteinases. These enzymes are a family of related zinc metalloenzymes which selectively degrade components of the extracellular matrix. We have isolated and characterized the matrix metalloproteinases produced by a variety of human tumor cell lines in culture and have demonstrated that the interstitial collagenase produced by many melanoma cells is identical to the

interstitial collagenase from human fibroblast cultures. Tumor cells often produce progelatinase A, a matrix metalloproteinase which selectively degrades type IV collagen, the major structural component of basement membranes. Our studies on the isolation of progelatinase A from human tumor cells led to the identification of a new member of the tissue inhibitor of metalloproteinases (TIMP) family, TIMP-2. TIMPs are endogenous and specific inhibitors of the matrix metalloproteinase enzymes and this property has been used to define this class of enzymes. TIMP-2 binds specifically to the latent gelatinase A through a specific C-terminal interaction and abolishes proteolytic activity of the enzyme following activation. We have cloned and sequenced TIMP-2 cDNA and localized the *Timp-2* gene to chromosome 17q25. Preliminary characterization of the gene structure reveals a 5' upstream region containing few known transcriptional regulatory elements and a rather simple gene structure consisting of four exons and three introns.

Immunohistochemical and *in situ* hybridization studies have demonstrated a strong correlation between the expression of gelatinase A and invasive behavior of many types of human tumors including human breast, colon, hepatocellular, thyroid, bladder cancers, and more recently, human brain tumors. A functional role for these enzymes in cell invasion was demonstrated by showing that inhibitors of these enzymes, including TIMPs, block tumor cell invasion both *in vitro* and *in vivo*. We have begun preclinical testing of synthetic metalloproteinase inhibitors in orthotopic models of metastatic human breast cancer to evaluate this approach for the treatment of human cancers.

Our studies on the invasive phenotype of human microvascular endothelial cells have demonstrated that this process is the result of a critical balance between activated metalloproteinase enzymes and their endogenous inhibitors the TIMPs. Thus, tumor-induced angiogenesis shares many functional similarities with the process of malignant tumor cell invasion. We have further explored the effects of TIMPs on human microvascular endothelial cells proliferation in response to the angiogenic cytokine basic fibroblast growth factor (bFGF). These experiments demonstrated that TIMP-2, but not TIMP-1, selectively blocked the mitogenic response of endothelial cells to bFGF. The use of the synthetic metalloproteinase inhibitor, BB94, confirmed that this effect was unique to TIMP-2 and was not mediated by metalloproteinase inhibitory activity of this protein. This is the first demonstration of a unique biological activity for TIMP-2 that is clearly independent of its ability to inhibit matrix metalloproteinases. Preliminary results suggest that these effects are mediated by a specific and saturable TIMP-2 receptor. Current efforts are aimed at isolation and characterization of this receptor.

Dr. David Kleiner has demonstrated the importance of the C-terminal domain of gelatinase A in the interaction of progelatinase A with TIMP-2 by cross-linking progelatinase A-TIMP-2 complexes before and after organomercurial activation. Dr. Kleiner has also demonstrated that TIMP-2 binding to progelatinase A stabilizes the complex against autoproteolytic degradation but does not prevent autoactivation of the protease following

exposure to organomercurial compounds. Dr. Kleiner is continuing to study the kinetic effects of TIMP-2 binding to gelatinase A on the activation, stability and substrate processing by this enzyme.

Dr. Jill Ray is studying the effects of genetic modulation of TIMP-2 expression on human melanoma cell attachment and spreading. The human A2058 melanoma cell line has been infected with LXSN retroviral constructs for both sense and antisense TIMP-2 transcripts. This has resulted in a selection of melanoma cell lines that both over- and under- express TIMP-2 with respect to the parental cell line. These clones demonstrate marked morphologic changes as well as changes in cell attachment, cell spreading and motility when compared with the parental A2058 cell line. Clones that both over- and under-express TIMP-2 have decreased *in vitro* and *in vivo* invasive activity. These preliminary results suggest that alterations in the matrix metalloproteinase and TIMP balance may disrupt not only ECM proteolysis but cell adhesion and migration required for effective cell invasion. Future experiments will be directed at understanding the coordination and interaction of cellular attachment, ECM proteolysis and cell migration to produce cellular invasion in both normal and neoplastic cells.

Women's Cancers Section

The Women's Cancers Section (WCS), under the direction of Dr. Patricia S. Steeg, performs basic molecular biology and biochemical investigations of women's cancers, primarily breast cancer. These studies may contribute to advances in cancer prognostics and the development of novel therapeutic approaches. Three major projects are under investigation in the WCS, described below.

The molecular basis of breast preneoplasia is the subject of the first WCS project. A significant number of lesions detected by mammography are premalignant hyperplasias or carcinomas *in situ*. These lesions signal an increased risk for subsequent development of an infiltrating carcinoma, and may represent precursor lesions. Molecular characterization of premalignant and *in situ* lesions have been hampered by a lack of cell lines, and the small size of biopsy lesions. Drs. Debra Weinstat-Saslow and Richard Manrow of the WCS, in collaboration with Dr. David Page of Vanderbilt University are studying the expression levels of three classes of genes, using *in situ* hybridizations on sections of formalin fixed, paraffin embedded lesions. These genes include (1) cell cycle control genes such as the cyclins; (2) growth factor receptor genes, and (3) DNA repair genes. The majority of work completed has addressed expression levels of cyclins A and D. While cyclins are normally expressed in association with the cell cycle, we have observed an apparent deregulation and overexpression of cyclin D in a proportion of atypical ductal hyperplasias and carcinomas *in situ*. Future experiments will determine if cyclin overexpression signals an increased risk for patient development of an invasive carcinoma, using banked tissue material with patient clinical course data from Vanderbilt University; transfection of each gene into a "normal" breast cell line will determine whether their overexpression is causally related to changes in proliferation rate,

apoptosis, genomic instability, etc. While still in its initial stages, this project combines pathology and molecular biology expertise to provide a comprehensive investigation of early breast neoplasia. Data generated may contribute to prognostic advances, and may also be used to develop therapeutic strategies for the prevention of progression to invasive cancer.

The second project in the WCS concerns the molecular regulation of angiogenesis, which is critical to both primary tumor growth and the establishment of distant metastases. We have observed that the extracellular matrix component thrombospondin (TSP) is quantitatively underexpressed in highly metastatic breast, lung and melanoma tumor cell lines. These data stand in agreement with published reports indicating that TSP inhibits endothelial growth and motility in angiogenesis, and suggest a negative regulatory role for TSP in the progression of certain cancers. To directly test this hypothesis, Dr. Debra Weinstat-Saslow in the WCS, in collaboration with members of the Biochemical Pathology Section, LP, has transfected the *thbs-1* cDNA into a human breast carcinoma cell line, and determined the phenotypic effects of TSP overexpression. The *thbs-1* transfectants exhibited reduced primary tumor size, metastatic potential and capillary densities *in vivo* and stimulated less endothelial motility *in vitro*, consistent with an anti-angiogenic function.

A potential anti-angiogenic portion of TSP has previously been ascribed to the central region of the protein, which contains heparin binding sites. However, we have characterized a mutant transfectant of MDA-MB-435 breast carcinoma cells which overexpresses a TSP protein missing 25 kDa of its C-terminus. This mutant transfectant retains full tumorigenicity and metastatic potential *in vivo*, and stimulates significant endothelial cell motility *in vitro*, suggesting an additional region of the TSP protein near its C-terminus to be critical to its anti-angiogenic function. Future experiments will define the critical domains of TSP using site directed mutagenesis and transfections. Subsequent experiments will test potential anti-angiogenic TSP peptides or recombinant protein domains for activity upon intravenous injection against unmodified MDA-MB-435 breast carcinoma and other human breast and ovarian carcinoma cell lines. This project is expected to identify a novel portion of TSP with anti-angiogenic activity, which may be suitable for therapeutic investigation to limit the metastatic outgrowth of breast and other cancers.

The third project of the WCS concerns the genetic regulation of the tumor metastatic process, centered on the *nm23* gene. The *nm23* cDNA was discovered by Dr. Steeg on the basis of its reduced expression in highly metastatic melanoma cell lines; reduced Nm23 expression has been correlated to lymph node metastases, poor differentiation grade and reduced patient disease free and overall survival in breast carcinoma cohorts. Similar trends linking reduced Nm23 expression to aggressive disease course have also been published in cohorts of human melanoma, gastric carcinoma and hepatocellular carcinoma, but do not extend to all cancer cell types. Transfection of murine *nm23*-1 into murine melanoma cells and human *nm23*-H1 into human breast carcinoma cells established that Nm23 overexpression can reduce *in vivo* tumor metastatic potential by 50-90%, with no significant effects on primary tumor size. *In*

vitro correlates of Nm23 overexpression include a reduced responsiveness to TGF-b in colonization assays, reduced motility to serum, IGF or PDGF in Boyden chamber assays, and morphological and biosynthetic evidence of breast ductal differentiation in matrigel cultures. Nm23 stands as one of a handful of metastasis suppressor genes described to date.

Drs. Nicholas MacDonald, Abel De La Rosa and Richard Manrow in the WCS are exploring three avenues of Nm23 investigation. First, the biochemical mechanism of Nm23 suppression is under investigation. The Nm23 protein has been proposed to have a multitude of biochemical properties, but convincing evidence implicating any of these activities in cancer metastasis is lacking. We have recently identified a novel, reversible phosphorylation of Nm23 on a serine residue. Levels of Nm23-serine phosphorylation, but not other Nm23 activities, were directly correlated with Nm23 expression levels and suppression of metastatic potential in transfection model systems. These data suggest the hypothesis that this serine phosphorylation pathway may be involved in Nm23's regulatory effects. Several experimental approaches are underway to confirm and extend these findings. Site directed mutagenesis of Nm23 will eliminate each of its proposed biochemical activities, and transfection of each mutated cDNA into breast carcinoma cells will determine the effect of each mutation on the metastatic phenotype. Proteins that specifically interact with Nm23, and may occupy upstream or downstream positions on its functional biochemical pathway, are under investigation. The data are expected to contribute to the identification of the functional biochemical pathway for Nm23 regulation of tumor metastatic potential.

The genomic organization and regulation of Nm23 gene expression are under investigation in the WCS. Data to date indicate that transcriptional regulation, and not allelic deletion of Nm23 is likely responsible for the reduced expression observed in aggressive breast carcinomas. Genomic clones for human *nm23*-H1 and *nm23*-H2 are under characterization. Promoter regions will be characterized, and tumor material will be examined for potential mutations. Also, any differences in the availability of transcriptional regulatory proteins will be investigated..

Given the transfection data indicating that Nm23 overexpression can negatively modulate the metastatic potential of certain tumor cell lines, we have hypothesized that elevation of Nm23 expression levels, its functional biochemical activity or downstream members of its functional biochemical pathway in tumor cells may be of therapeutic benefit for limiting metastatic spread. In collaboration with the Developmental Therapeutics Program, DCT, NCI, we have identified 45 pharmacological agents from a bank of 32,000 which are preferentially cytostatic/cytotoxic *in vitro* on low Nm23 expressing human breast and melanoma tumor cell lines. Planned investigations will determine the *in vitro* effects of each agent on Nm23 expression and biochemistry, and effects on standard metastasis related *in vitro* assays using a panel of breast and ovarian carcinoma cell lines. These data will be used to prioritize agents for *in vivo* testing, and eventual consideration for clinical trials.

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.

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.

Signal Transduction and Prevention Unit

This Unit, under Dr. Elise Kohn, is divided into basic science investigation, translational studies, and clinical trials. It has the mission to define the role of transmembrane calcium-mediated signal transduction in cancer development, proliferation, dissemination, and prevention; to identify new genes which are involved in calcium-mediated signal transduction pathways and apply the study of those genes to cancer prevention; to use this knowledge for the development of novel therapeutics for treatment and prevention of cancer proliferation and dissemination; and to conduct the pilot clinical trials of novel therapeutics and combinations.

The basic science studies are focused on the sites of action of calcium-mediated signaling on prevention and invasion using cellular and molecular biologic techniques. Structure function analysis of a novel inhibitor of non-voltage gated calcium influx has yielded a family of compounds. Study of this family of analogs has demonstrated a concordant inhibition of calcium influx and proliferation. These compounds are now the tool for purification and characterization of the CAI binding site(s). CAI resistant cells of three independent malignant cell types have been established. Subtractive hybridization has identified several genes which are overexpressed in the

resistant cell line and are the object of ongoing work. Collaborative studies have used a novel inhibitor of non-voltage gated calcium influx to study regulation of specific genes including gelatinase A (MMP-2), VL-30, and c-*fos*.

Translation of the basic science findings to related basic or clinical science questions bridge the way to direct clinical investigation of the identified therapeutics and include application of pathway modulation to biologic endpoints such as angiogenesis and invasion, as well as preclinical development. Data demonstrate CAI inhibited gelatinase A gene expression and both showed a marked inhibition of the component parts of angiogenesis and inhibition of *in vivo* angiogenesis. Studies have been initiated to evaluate other important cellular signaling pathways such as calcium-mediated tyrosine phosphorylation, to investigate the use of inhibition of calcium-mediated signaling to augment the efficacy of key chemotherapeutic agents, such as paclitaxel, and for preclinical development of CAI as a chemopreventive agent.

The clinical arm is charged with clinical trials design, pharmacokinetic analysis, and metabolite identification and has been instrumental in the development, design, execution, and analysis of the first clinical trial of a signal transduction therapy agent. The clinical trial of the parental CAI compound opened for accrual in March of 1992 and is expected to be completed shortly. The Unit has done all of the plasma and urine CAI levels for pharmacokinetic analysis and is developing a method for CAI tissue level analysis. The Unit has completed the design of a Phase I protocol of paclitaxel with CAI for submission and initiation. Plans include Phase II studies of CAI as a single agent, further investigation of its role in combination, and initiation of CAI adjuvant and prevention trials.

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.

The section studies differential gene expression in neoplasia. Adhesion, proliferation, signal transduction, and transcriptional regulation of tumor cells are the focus of several studies to understand the biology of cancer cells and to develop potential molecular markers of diagnostic and prognostic value.

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. 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.

To study a potential molecular marker for proliferation, a complete cDNA sequence for the catalytic subunit of human ribonucleoside reductase has been cloned. This enzyme catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates and is a rate limiting step of DNA synthesis. The catalytic subunit is expressed only during the S phase of the cell cycle and levels of mRNA are increased in colorectal carcinomas compared to matched normal adjacent colonic tissue. The data are consistent with the hypothesis that catalytic subunit mRNA is increased to provide rate limiting precursors for DNA synthesis in rapidly proliferating colonic cancer cells. Current plans are to study the expression of this gene in a variety of human cancers and to determine if it may serve as a useful molecular marker for proliferation.

As a potential indicator of calcium-dependent cellular processes, the calmodulin I gene is being analyzed. The calmodulin I gene expresses two mRNA transcripts of approximately 1.6 and 4.4 kb. The mechanism for the presence of the two transcripts has been determined to be the alternate use of polyadenylation signals in the gene. Furthermore, regulatory sequences in the 3' untranslated region of the longer mRNA have been identified that may control its degradation. Unexpectedly calmodulin I mRNAs are down regulated in colorectal carcinomas. The expression of calmodulin I mRNA will be studied in other human cancers to assess the utility of developing calmodulin I as a molecular marker for transformed cells.

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 studies 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 of the breast cancer homeobox genes contain alterations compared to previously described homeobox sequences, including a unique alternate splicing site in HOX C6, and a polymorphism in HOX B7 resulting in a potential change in the length of the homeobox protein. Current studies are designed to assess the physiologic significance of these alterations and to study the expression of the various HOX genes in human breast cancers by *in situ* hybridization.

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.

Cytopathology Section

The Cytopathology Section provides complete diagnostic service in cytopathology for the Clinical Center of the National Institutes of Health. We utilize ancillary diagnostic techniques such as immunocytochemistry, flow cytometry or electron microscopy when appropriate, to confirm interpretations made by routine light microscopy or enhance cytological diagnostic accuracy. In medical practice today, cytopathology is no longer simply a screening modality, but rather provides definitive diagnoses which direct patient care and treatment.

The Cytopathology Section evaluates approximately 3,8000 specimens per year. The overall caseload has been increasing by approximately 5% per year over the past 5 years. Approximately 15% of specimens are cervical/vaginal smears, 14% are FNA specimens and approximately 15% of cases overall are diagnostic of malignancy. The number of cases received from outside pathologists for consultative review has increased over the past 5 years from just under 100 per year in 1989 to 160 in 1993. Although the absolute number of specimens is not high, the relatively high rate of pathologic findings combined with the diversity of types of exfoliative and FNA specimens, provides a broad experience in diagnostic cytopathology for residency and fellowship training.

The Cytopathology Section is involved in several clinically-related research projects, many of which utilize fine needle aspiration (FNA) and immunocytochemistry to provide ancillary diagnostic information regarding expression of various tumor antigens, HLA antigens or other markers. A partial listing of such studies includes: (1) Evaluation of percent of tumor cells expressing CD22 in FNA specimens as part of a clinical trial using monoclonal antibody therapy for refractory CD22 positive lymphomas; (2) Evaluation of expression of drug resistance marker P-glycoprotein prospectively by FNA in breast cancer patients treated with new combination chemotherapy; (3) Determination of expression of HLA Class I antigen expression in patients with metastatic melanoma treated with gamma interferon and IL-2.

Postmortem Pathology Section

The Postmortem Pathology Section oversees the performance of autopsies by residents as well as resident education in autopsy pathology. It also monitors the research use of archival autopsy material as well as approving

requests for fresh autopsy tissues. In addition to projects from outside the Laboratory of Pathology which use autopsy material, both archival and fresh autopsy tissue is used by the staff and residents of the Laboratory of Pathology in ongoing studies. These projects include traditional clinicopathological correlative studies as well as investigations into the basic molecular pathophysiology of the diseases studied at the Clinical Center. Electron microscopic, immunohistochemical, molecular and flow cytometric studies are used to enhance our understanding of autopsy findings.

Dr. David Kleiner, working in collaboration, with Dr. William G. Stetler-Stevenson, is engaged in investigating the basic biochemical interactions between the 72 kDa type IV collagenase (gelatinase A) and tissue inhibitor of metalloproteinase-2 (TIMP-2). He also carries out collaborative projects with the Liver Diseases Section of the NIDDK correlating changes in liver histopathology following the treatment of chronic liver disease and with the Surgery Branch of the NCI characterizing the effects of isolated liver perfusion with tumor necrosis factor alpha on both normal animals and human subjects with liver metastases.

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. More than two thirds of them (approximately 150 cases) are completed and supplemental Electron Microscopy (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. Mechanisms of tumor development in solid poorly differentiated pediatric tumors:

A) Rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma and primitive neuroectodermal tumors (PNET) are investigated for the presence of p53 mutations. Polymerase chain reaction (PCR)-amplified DNA from paraffin blocks is analyzed by mutation detection enhancement (MDE) gels and/or single stranded conformation polymorphism (SSCP). The presence of mutations is verified by direct DNA-sequencing.

B) Levels of mRNA and protein expression, as well as possible DNA amplification for the mdm-2 gene which has been proposed as an alternative pathway for p53 gene inactivation are also investigated in the same groups of tumors.

2. Growth factors and genes (fas/bcl2) as potential treatment modalities in pediatric tumors:

A) The role of Transforming Growth Factor (TGF)-β in the growth and differentiation of rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma and PNET is studied *in vitro*.

Cell growth and differentiation after addition of TGF-β is evaluated by cell proliferation assays, levels of expression of muscle genes, such as creatine kinase MM, by Northern blot analysis, and morphologic ultrastructural studies. Affinity of the growth factor for its own receptors is evaluated by binding assays and TGF protein levels are measured in tissue culture media with an enzyme-linked immunosorbent assay and CCL-64 mink lung cell growth inhibitory assay.

B) The possibility of manipulating programmed cell death (apoptosis) in pediatric round cell tumors is studied by investigating the quantitative and qualitative status of fas and bcl-2 antigens.

Variable but generally high expression of fas antigen is found in Ewing's/PNET cell lines by immunofluorescence and flow cytometry. The role of this antigen in apoptosis is studied by: 1) reverse transcriptase PCR for detection of specific apoptosis-related DNA sequences and/or mutations and 2) evaluation of induced tumor cell death after addition of anti-fas antibody in culture media at various time intervals. Cell death is evaluated by flow cytometry of propidium iodine-stained cells.

3. Diagnostic and prognostic use of PCR and *in situ* hybridization techniques:

A) Detection of specific fusion transcripts in tumors with 11;22 translocation by reverse transcriptase-PCR leads to specific diagnosis of Ewing's/PNET.

B) Trisomy of chromosome 1 has been recently implicated in clinical aggressiveness of Ewing's/PNET. Using probes for chromosome 1 the chromosomal 1 copies are studied in Ewing's/PNET nuclei by fluorescence *in situ* hybridization (FISH). Clinical correlations will follow.

4. Immunohistochemical studies:

Combination of the 12E7 antibody against antigenic surface determinants of Ewing's and PNET cells with the antibody against the muscle determination gene protein $MyoD_1$ offers specificity and sensitivity in the differential diagnosis of primitive rhabdomyosarcoma from Ewing's sarcoma and PNET.

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 NIAMSD. 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 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 angiocentric lymphomas. Evidence of an association with the Epstein-Barr virus (EBV) has been extended to include a cluster of cases in Peru. As described previously in Asian countries, angiocentric lymphomas in Peru frequently present with midline destructive nasal disease. EBV was identified by *in situ* hybridization in all cases expressing a T-cell phenotype. More recent work has suggested that the nasal and pulmonary forms of angiocentric lymphoma may be distinct. Both are

associated with EBV. However, in the pulmonary form EBV was localized in B cells, which were associated with an exuberant reactive T-cell infiltrate. By contrast, in nasal lesions EBV is found in T/NK cells.

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.

Specialized Diagnostics Unit

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 may 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 precede histologic progression and may 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 discovered that most Burkitt's lymphomas are 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. Work continues to define the biologic significance of these mutations and their potential contribution to lymphoma genesis.

Flow Cytometry Unit

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 some 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 Cherynobl 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.

Dr. Stetler-Stevenson is studying the effects of innovative therapies for lymphoma in collaboration with the Medicine Branch and Pediatric Branch, DCT, NCI. Her laboratory monitors the biological response of patients with B-cell neoplasms receiving immunotoxin therapy. Flow cytometric evaluation of peripheral blood has documented a diminution of tumor cells without a detrimental effect on the immune system. Moreover, she has observed that patients with circulating malignant cells are partially protected from the vascular leak syndrome, a serious side effect of immunotoxin therapy. This protective effect is seen even when peripheral involvement is minimal and constitutes less than 1% of the lymphocyte population. These data will be used in a new immunotoxin protocol to attempt to select patients at low risk for toxicity for inclusion in a high dose treatment regimen. Flow cytometric analyses have also revealed that some patients who develop the vascular leak syndrome have a prominent population of degranulated neutrophils in the peripheral blood prior to clinical signs of toxicity.

Dr. Stetler-Stevenson has also studied bone marrow aspirates from pediatric patients with acute leukemia to determine the presence of minimal residual disease based upon the identification of cells with unique and abnormal immunophenotypes. These immunophenotypic data are correlated clinical course.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00853-41 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

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
+L. Middleton	Medical Staff Fellow	LP NCI
+G. Oliver	Medical Staff Fellow	LP NCI
+J. Wilson	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.

A variety of histologic, immunohistology and molecular biology studies are being performed by the surgical pathology staff in breast, thyroid, colon and kidney cancer.

+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:

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.

Batista M, Cartledge T, Zellmer A, Merino MJ, Axiotis C, Loriaux L, Nieman L. Delayed endometrial maturation induced by daily administration of the progesterone antagonist RU486: A potential novel contraceptive strategy. Am J Obstet Gynecol 1992;167:60-4.

Monterroso V, Jaffe E, Merino MJ, Medeiros J. Ovarian malignant lymphoma. A clinicopathologic analysis of 39 cases. Am J Surg Pathol 1993;17:154-70.

Sandrock D, Merino MJ, Norton J, Neumann R. Ultrastructural histology may explain results of TI-201/Tc-99m parathyroid subtraction scintigraphy. J Nucl Med 1993;34:24-9.

Merino MJ, Jaffe G. Age contrast in ovarian pathology. Cancer 1993;71:537-44.

Hollingsworth H, Pogrebniack H, Baker A, Merino MJ. Benign mesenchymoma of the buttock. Am Soc Clin Pathol 1993;TD93-1 (TD 117).

Gilbert L, Elwood L, Merino MJ, Masood S, Barnes R, Lazarous D, Townsend A, Cowan K. A pilot study of Pi class glutathione S-transferase (GST) expression in breast cancer: Correlation with estrogen receptor and prognosis in node-negative breast cancer. J Clin Oncol 1993;11:49-58.

Batista MC, Cartledge TP, Merino MJ, Platia MP, Loriaux L, Nieman LK. Midluteal phase endometrial biopsy does not accurately predict luteal function. Fert Steril 1993;59:294-300.

Gomella L, Ehya H, Rupp M, Merino MJ, Robertson C, Linehan M. Flow cytometric DNA analysis of interleukin-2 responsive renal cell carcinoma. J Surg Oncol 1993;53:252-5.

Kragel P, McClellan W, Pestaner J, Merino MJ. Sarcomatoid renal cell carcinoma: five cases with immuno- and lectin histochemistry supporting proximal tubular origin. Int J Surg Pathol 1993;1:107-10.

Hollingsworth H, Merino MJ, Baker A. Unusual mesenchymoma with prominent chondro-osseous elements. Int J Surg Pathol 1993;1:129-34.

Johnstone P, Pierce L, DeLaney T, Merino MJ. Primary soft tissue sarcomas of breast. Int J Radiat Oncol Biol Phys 1993;27:671-5.

Prewitt TW, Lubensky IA,Pogrebniak HW, Pass HI. Orthotopic implantation of mesothelioma in pneumonectomized immune-deficient rat: a model for innovative therapies. Int J Cancer 1993;55:877-80.

Pogrebniak HW, Lubensky IA, Pass HI. Differential expression of platelet derived growth factor-b in malignant mesothelioma: a clue to future therapy? Surg Oncol 1993;2:235-40.

Ritland F, Lubensky IA, LiVolsi VA. Polymorphous low-grade adenocarcinoma of the parotid salivary gland. Arch Pathol Lab Med 1993;117:1261-3.

Canete-Soler R, Litzky LA, Lubensky IA, Muschel RJ. Localization of the 92 kDa gelatinase mRNA in squamous cell and adenocarcinomas of the lung using in situ hybridization. Am J Pathol 1994;144:518-27.

Stern SB, Peck GL, Haupt HM, Hollingsworth H. Malignant melanoma in xeroderma pigmentosum: search for a precursor lesion. J Am Acad Dermatol 1993;28:591-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09145-10 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

As described previously, subspecialty expertise in diagnostic neuropathology is provided to the Laboratory of Pathology, NCI, and to all other institutes, via the Office of the Clinical Director, 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 approximately 1/2 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 75 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 (NCI).

4. HTLV-1-associated myelopathy: clinicopathologic study, correlation with PCR (NCI, NINDS).

5. Experimental treatment of malignant brain tumors: gene therapy, immunotoxin (NINDS); pediatric tumors (NCI).

Publications:

Navarro-Roman L, Roman 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)

Schiffmann R, Moller JR, Trapp BD, Shih HH-L, Farrer RG, Katz DA, Alger JR, Parker CC, Hauer PE, Kaneski CR, Heiss JD, Kaye EM, Quarles RH, Brady RO, Barton NW. Childhood ataxia with diffuse central nervous system hypomyelination. Ann Neurol 1994;35:331-40.

Katz D, Taubenberger JK, Cannella B, McFarlin DE, Raine CS, McFarland HF. Correlation between magnetic resonance imaging findings and lesion development in chronic, active multiple sclerosis. Ann Neurol 1993;34:661-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09361-04 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

p53 Mutations in Breast Cancer with C-erbB2

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:	T. Castiglioni	Visiting Fellow	LP NCI
	B. Bryant	Medical Technician	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:	PROFESSIONAL:	OTHER:
3	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.)*

Mutations of the p53 suppressor gene have been shown to be potentially useful as a prognostic indicator of tumor aggressiveness. We will study 85 cases of breast cancer utilizing immunohistochemistry and will correlate the expression of this protein with amplification of c-erbB2 protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09380-02 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	

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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.)*

Histological evaluation of biopsies in premenopausal patients with beast 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, and 4) to compare histologic changes with those of elderly females. Prognostic markers, such as cerb-2, p53 and nm23 will be studied by immunohistochemistry and *in situ* hybridization in order to determine their role in prognosis and to compare them with breast cancer in the lederly population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09391-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Quantification of Glucose Transporter Proteins in Breast Lesions

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

PI:	R. Neumann	Chief, Nuclear Medicine	NM CC
OTHER:	M. Merino	Chief, Surgical Pathology Section	LP NCI
	L. Elogi	Visiting Fellow	LP NCI
	B. Bryant	Medical Technician	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:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

By immunohistochemistry and quantitative *in situ* hybridization techniques, we will correlate these findings with glucose update as measured by PET scanning techniques. This study provides the foundation for studying the *in vivo* changes of breast lesions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09392-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Determination of Genetic Mutations in Atypical and Cancerous Breast Lesions

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:	E. Moro	Visiting Fellow	LP NCI
	M. Sobel	Chief, Molecular Pathology Section	LP NCI
	L. Liotta	Chief, Tumor Invasion and Metastases 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:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

It is still unclear the type of genetic alterations that patients with breast cancer have and (1) whether these alterations are different or similar to patients with familial history of breast and (2) whether these changes occur in pre-malignant lesions.

Utilizing paraffin-embedded material and following the technique described by Sukpanichnant, we will search by PCR amplification for a variety of genetic alterations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09393-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Evaluation of Genetic Alterations in Neoplastic and Pre-Neoplastic Gastric 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:	J. Sanz Ortega	Visiting Fellow	LP NCI
	M. Sobel	Chief, Molecular 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:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Utilizing paraffin-embedded material and the PCR technique, we will be searching for a variety of genetic mutations that may or may not be expressed in gastric cancer and some of the pre-neoplastic lesions, and we will correlate with clinical outcome as well as with other known prognostic factors such as p53 and C-erbB2.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09364-03 LP

PERIOD COVERED
October 1, 1993 to September 30, 1994

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: D. Kleiner Acting Chief, Postmortem Pathology Section LP NCI

OTHER: C. Baker, D. Levens, L. Liotta, S. Mackem, T. O'Leary, A. Ginsberg, J. Taubenberger, K. Gardner, S. Barksdale, M. Buck, M. Roth, L. Cheshire, J. Teruya, C. Phillips, Z. Zhuang, W. Stetler-Stevenson, I. Lubensky, L. Abruzzo, D. Katz, W. Travis, A. Samad, L. Middleton, G. Oliver, J. Wilson, M. Jerome, A. Dock, J. Rainey, W. Young

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Postmortem Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
29	25	4

CHECK APPROPRIATE BOX(ES)

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

A

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

The 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: clinicopathological studies in dementia; MRI correlations with normal tissue and demyelinating disease (multiple sclerosis); experimental therapy of malignant brain tumors (both primary and metastatic); evaluation of cellular adhesion molecules involving organs in interleukin-2 treated individuals; normal tissues used for purification of antigens to make antibodies; use of autopsy materials as quality control tissue for immunohistochemistry; distribution of HIV at the time of death; correlation of HIV disease/sequelae with quinolinic acid concentration in neural tissue; distribution of non-muscle myosin heavy chain in neural and vascular tissues, renal tissue controls for diabetic kidney disease.

A database of major autopsy findings from 1953 through the present is being compiled, with pertinent historical information included. The database is approximately 15-20% complete.

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 00852-41 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	O. Garza	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 BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

A

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

The Cytopathology Section provides complete diagnostic service in exfoliative cytology and fine needle aspiration cytology. The section also routinely applies immunocytochemistry techniques to confirm and/or enhance cytological diagnostic accuracy. In addition, the section collaborates in various clinical research projects utilizing routine microscopy as well as special staining techniques and immunocytochemistry.

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.

The Cytopathology Section initiates and collaborates in various clinical research projects. A partial listing of recent and/or ongoing projects includes: 1) Diagnosis of pulmonary microvascular tumor embolization by cytologic evaluation of pulmonary artery catheter blood samples; 2) Role of cytopathology in tumor infiltrating lymphocyte (TIL) immunotherapy; 3) Respiratory cytology in chronic granulomatous disease; 4) Fine needle aspiration of thyroid; 5) Cytopathology of atypia in atrophy.

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% 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 over the past 2 years 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:

Solomon D. Fine needle aspiration of the thyroid: An update. Thyroid Today 1993;26(3).

Abati A, Landucci D, Danner RL, Solomon D. Diagnosis of pulmonary microvascular metastases by cytologic evaluation of pulmonary artery catheter derived blood specimens. Hum Pathol 1994;25:257-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00897-11 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Diagnostic Adjuncts 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:	A. Abati	Deputy Chief, Cytopathology Section	LP NCI
	J. O'Shaughnessy	Medical Officer	COP DCT NCI
	K. Cowan	Senior Investigator	COP DCT NCI
	E. Sausville	Chief, Lab. of Biol. Chem.	DCT NCI
	D. Danforth	Senior Investigator	DCT 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:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

A

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

Immunocytochemistry has proved to be a valuable diagnostic adjunct to cytopathologic diagnosis. The Cytopathology Section is involved in several clinically-related research projects, many of which utilize fine needle aspiration (FNA) and immunocytochemistry to provide ancillary diagnostic information regarding expression of various tumor antigens, HLA antigens or other markers. A partial listing of such studies includes: (1) Evaluation of percent of tumor cells expressing CD22 in FNA specimens as part of a clinical trial using monoclonal antibody therapy for refractory CD22 positive lymphomas; (2) Evaluation of expression of drug resistance marker P-glycoprotein prospectively by FNA in breast cancer patients treated with new combination chemotherapy; (3) Ultrasound guided FNA of parathyroid lesions; (4) Cytologic and immunocytochemical characterization of mantle cell lymphomas; (5) Cytologic diagnosis and immunophenotyping of ocular lymphomas.

Major Findings:

The cytological diagnosis of malignant lymphoma can be extremely difficult because the cytological features of the malignant cells in small cell and mixed small and large cell lymphomas may be indistinguishable from those of reactive lymphoid cells. We have examined the usefulness of the avidin biotin immunoperoxidase technique and a battery of antibodies to T and B cell markers to the diagnosis of lymphoma in cytological specimens. We conclude that immunocytochemistry is very useful in the cytological diagnosis of non-Hodgkin's lymphoma. Further, it is possible to diagnose the vast majority of lymphomas using only the immunoglobulin light chain markers κ and λ and the T-cell markers 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.

Publications:

Abati A, Skarulis M, Shawker T, Solomon D. Ultrasound-guided FNA of parathyroid lesions: A morphologic and immunocytochemical approach. Hum Pathol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09176-06 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

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) drafting of "The National Strategic Plan for Breast and Cervical Cancer - Cervical Cancer Quality Assurance"; 3) development of "Interim Guidelines for Management of Abnormal Cervical Cytology"; 4) numerous projects in conjunction with CDC's National Breast and Cervical Cancer program.

I have also acted as a consultant for a National Library of Medicine project to capture and electronically seam together microscopic images to create a computer simulation of microscopic screening. Mr. Earl Henderson and I were recently invited to discuss this work at a CDC workshop on proficiency testing in cytology. This technology has tremendous potential educational applications; unlike static images, this technique can be used to teach and measure detection skills.

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 co-edited theTBS reference atlas which includes morphologic criteria and accompanying photomicrographs.

2) 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.

3) 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.

I field over 100 public and press inquiries per year for information or responses to specific issues. 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 asssurance 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.

Recently, at the request of the American Cancer Society, I hosted a public question and answer forum in New England on cervical cancer screening. The forum was organized in response to public concern arising from the death of a prominent public official due to cervical cancer.

Publications:

Solomon D. Screening for cervical cancer: Prospects for the future. (Editorial) J Natl Cancer Inst 1993;85:1918-9.

Solomon D. Nomenclature for cervicovaginal cytology. In: Keebler CM, Somrak TM, eds. The Manual of Cytotechnology, 7th Edition. Chicago: ASCP Press, 1993, pp. 79-86.

Kurman RJ, Solomon D, eds. The Bethesda System for Reporting Cervical/Vaginal Cytologic Diagnoses: Definitions, Criteria, and Explanatory Notes for Terminology and Specimen Adequacy. New York: Springer-Verlag, 1994.

Henderson E, Seamans JF, Solomon D. Electronic conversion of microscopic images from cytologic glass slides. Lab Med 1994;25:264-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09385-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Morphologic, Immunocytochemical and F.I.S.H. Analyses in Cytology

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

PI:	A. Abati	Deputy Chief, Cytopathology Section	LP NCI
OTHER:	P. Fetsch	Medical Technologist	LP NCI
	D. Solomon	Chief, Cytopathology Section	LP NCI
	O. Garza	Biotechnology Fellow	LP NCI
	Y. Hijazi	Medical Expert	LP NCI
	A. Cajigas	Biotechnology Fellow	LP NCI
	A. Wilder	Cytotechnologist	LP NCI

COOPERATING UNITS *(if any)*

H. Pass, NCI; D. Danforth, NCI; M. Skarulis, Metabol. Disease Branch, NIH; T. Shawker, Diagostic Radiology Dept., Clin. Ctr., NIH, Z. Ram, Surg. Neurol. Branch; W. Jaffurs, Cytology Services of Maryland, S. Wolman, ONCOR, Inc.

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.65	.4	.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

In the last year, the focus of my work has been on morphologic and immunocytochemical analyses of several focused areas of cytology. We have shown through the immunocytochemical characterization of mesothelioma cell lines, that these cells when cultured retain their in situ immunocytochemical characteristics and that the most reliable preparation for immunocytochemistry in cytologic samples of mesothelioma is the cell block. In an attempt to further enhance our battery of markers for mesothelioma, we evaluated a recently developed antibody, OV 632, which we proved to have a low specificity despite preliminary reports to the contrary. Other cell line characterization work included evaluation of breast adenocarcinomas for estrogen and progesterone receptors. Several projects which I have completed have more direct clinical applicability. Through the use of morphology and limited immunocytochemistry, we were able to expeditiously identify with certainty parathyroid tissue obtained through ultrasound-guided FNA for preoperative localization in hyperparathyroid patients. Previously, this determination was dependent on an RIA not performed at our institution. We described the cytologic effects of photodynamic therapy in body fluids and reported respiratory cytology in chronic granulomatous disease. Ongoing projects include: evaluation of the morphologic spectrum of atrophy in PAP smears in an attempt to identify true preneoplastic changes; evaluation of optimal specimen preparation for fluorescence in situ hybridization in cytology; and analysis of cerebrospinal fluid in intrathecal gene therapy for the treatment of leptomeningeal carcinomatosis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09354-04 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Transforming Growth Factor (TGF)-ß 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 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:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

TGF-β1 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 *in vitro* without affecting cell proliferation. It has been postulated that TGF-β1 may have a role in muscle regeneration and may prevent precocious fusion of embryonic myoblasts. The role of TGF-β in myogenous tumors is unknown. Using the avidin-biotin immunoperoxidase technique, we found that rhabdomyosarcomas (RMS) stain intensely for TGF-β1 and TGF-β3, but not TGF-β2 *in vivo*.

This project aims at investigating the possible role of TGF-β in the growth and differentiation of RMS *in vitro*. Three established RMS cell lines (RD, Birch, and RH18) were selected for the studies.

Major Findings:

Using the 3H-thymidine incorporation assay, we found that TGF-β1 at concentrations of 0.25 to 1 ng/ml inhibits cell growth in all 3 RMS cell lines grown in serum free media (40-75% inhibition). This inhibitory effect appeared to be specific to exogenous, but not endogenous TGF-β1 with blocking antibody experiments. Since TGF-β1 mRNA was detected in all 3 RMS cell lines and increased after treatment with TGF-β1 in 2 of them, suggesting an autocrine role for TGF-β1, we hypothesized that lack of detection of endogenous TGF-β1 function may be due to an inactive form of endogenous TGF-β1, which is usually the case in tumor models *in vitro*. This was confirmed with the mink lung fibroblast (CCL-64) bioassay which showed no active form of TGF-β isoform *in vitro*.

Cell growth inhibition was independent of cellular differentiation. The latter was evaluated with a cell fusion assay, ultrastructurally, and with Northern blotting of mRNA levels for the muscle proteins desmin and creatine phosphokinase MM, as well as the muscle determination genes MyoD1 and myogenin.

This project is very close to completion. We are currently investigating the duration of the growth inhibitory effect of TGF-β (temporary versus permanent) with clonogenic assays.

Publications:

McCune BK, Patterson K, Chandra RS, Kapur S, Sporn M, Tsokos M. Expression of transforming growth factor-β 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 09370-03 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Biology of 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	Visiting Fellow	LP NCI
	J. Keleti	Visiting Fellow	LP NCI
	S. Mims	Biologist	LP NCI
	J. Jefferson	Medical Technologist	LP NCI
	R. Gosnay	Special Volunteer	LP NCI

COOPERATING UNITS *(if any)*

G. Tsokos (Assistant Chief), DCI, WRAMC; T. Szentendrei (Fellow), DCI, WRAMC; D. Vassilopoulos (Fellow), DCI, WRAMC

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	3	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Tumors similar to normal tissues preserve their homeostasis via a delicate interplay between cell proliferation and cell death. Both events are controlled genetically by various genes. We chose to study the p53 suppressor gene and its inhibitory gene MDM2, as well as the apoptosis related genes fas and bcl2 in Ewing's sarcoma (ES)/Peripheral Primitive Neuroectodermal Tumors (PNET), neuroblastoma (NB) and rhabdomyosarcoma (RMS).

(a) p53/MDM2 genes in pediatric tumors: The presence of p53 mutations was studied in 13 RMS, 10 ES/PNET and 3 NB tissues and in 9 RMS, 7 ES/PNET, 7NB and 1 olfactory NB cell lines. The tissues consisted of archival paraffin-embedded and frozen material. All tissues and cell lines were screened for the presence of mutations with the single-strand conformation polymorphism (SSCP) method. The presence or absence of mutations was further confirmed with direct cDNA sequencing.

(b) Fas/Bcl2 genes in ES/PNET and NB: Seven ES/PNET, 5 NB and 1 olfactory NB cell lines were studied for the presence of Fas antigen by flow cytometry and immunoperoxidase, as well as their response to an anti-Fas cytotoxic antibody. Cytolygic assays were performed after incubation of the cells with 333 ng/ml of anti-Fas antibody at 37°C for 17 hr. Cell death was evaluated with propidium iodine staining and an Epics Elite flow cytometer equipped with an Argon laser (Coulter). Possible alterations of the Fas gene were studied by direct DNA sequencing of reverse transcription (RT)-PCR products using several primers spanning the cytoplasmic and intracellular portion of the Fas cDNA.

Levels of expression of the bcl2 gene will also be investigated in all tumor cell lines.

Bcl2 gene staining of tumor tissues from homogeneously treated ES/PNET patients with nonmetastatic disease will also be performed and correlation of levels of bcl2 expression with patient survival will be statistically analyzed.

Major Findings:

(a) p53 mutations were found in 8/13 cases (63%) of RMS tumor tissues and involved exon 7 in 6/8. ES/PNET tumor tissues showed very low frequency of p53 mutations (15.4%) and NB lacked p53 mutations. A significantly higher incidence of p53 mutations was observed in ES/PNET cell lines (100%), but not in RMS and NB cell lines. These data suggest selective growth advantage of p53 mutant ES/PNET cells in vitro. The MDM2 gene, whose product may form complexes with the p53 protein, thus inhibiting its function, was found highly expressed in all 3 RMS cell lines lacking p53 mutations and not in any other line or the remaining RMS cell lines with p53 mutations. In one RMS cell line, the high MDM2 mRNA and protein expression was associated with amplification and rearrangement. This inverse correlation of the p53 and MDM2 genes in RMS and lack of correlation in the other strudied pediatric tumors suggests that alterations of the p53 pathway are significant in the development of childhood RMS.

(b) All ES/PNET cell lines expressed variable but detectable levels of the Fas antigen on the cell surface; all NB cell lines lacked Fas antigen from the cell surface. Cytoplasmic staining for Fas was observed in all studied tumor cell lines.

All but 2 PNET/ES cell lines responded to the anti-Fas antibody with cell death, in contrast to all NB cell lines which remained nonresponsive to the anti-Fas antibody. Furthermore, ES/PNET cell lines have shown no alterations of the Fas gene, but similar experiments in the NB cell lines are suggestive of a missing region in the proximal cytoplasmic portion of the Fas cDNA. We are currently in the process of further characterizing this missing region in NB cell lines using various sets of primers.

The establishment of this in vitro model to manipulate apoptosis in pediatric tumors offers attractive possibilities for novel therapeutic approaches.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09394-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	M. Quezado	Visiting Fellow	LP NCI
	S. Mims	Biologist	LP NCI
	J. Jefferson	Medical Technologist	LP NCI
	C. Brown	Biol. Lab. Technician	LP NCI
	R. Gosnay	Special Volunteer	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:	PROFESSIONAL:	OTHER:
6	3	3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The Ultrastructural Pathology (UP) Section has the following functions: (A) It provides specialized diagnostic services for cases difficult to classify with conventional methods. (B) It provides diagnostic consultative services to the Pediatric Oncology Branch (POB) at the National Cancer Institute (NCI) and participates in a weekly Tumor Board and (C) It is engaged in independent and collaborative research with the ultimate goal 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.

The Section's research projects with diagnostic application consist of: (a) The use of the transcription polymerase chain reaction (RT-PCR) to detect abnormal transcripts in tumors with specific chromosomal translocations:

Recent cloning of the breakpoints of the t(11;22) (q24;q12) and t(2;13) (q35;q14) translocations which are encountered in Ewing's sarcoma/Peripheral Primitive Neuroectodermal Tumors (PNET) and alveolar rhabdomyosarcoma (RMS) respectively, has made possible the detection of specific fusion transcripts with RT-PCR. We used published sets of primers flanking those translocations and the RT-PCR technique and studied 17 tumor cell lines and 25 tumors, most of which were archival paraffin-embedded material.

(b) The use of MyoD1 and 12E7 as specific markers for the diagnosis of RMS and Ewing's sarcoma/PNET respectively.

An antigen retrieval method consisting of incubation of tissue sections in a microwave oven in citrate buffer, has enabled us to successfully detect the presence of MyoD1 in formalin-fixed, paraffin-embedded tissues.

The 12E7 antibody is directed against the mic2 protein which is a human T-cell surface glycoprotein, and which has been reliably and specifically detected in Ewing's sarcoma and PNET and considered to be a marker for these tumors. We stained 38 Ewing's sarcoma/PNET and 45 RMS cases to evaluate the role of this protein in the differential diagnosis of Ewing's sarcoma/PNET from poorly differentiated RMS.

Major Findings:

Diagnostic Data:

In 1993 174 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 turn-around time for completion of each case varies according to the clinical needs. Some cases are processed and photographed in 24 hrs. (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.

Pathology material from 76 pediatric patients was reviewed and presented in the POB Tumor Board by the UP Section's professional staff in the year of 1993.

Clinically Applied Research Data:

The UP Section maintains a frozen tissue bank and various tumor cell lines from pediatric tumors. The frozen tissues serve as material for diagnostic and/or research studies. The data from the RT-PCR analysis of pediatric tumors have shown that this method can reliably identify Ewing's sarcoma/PNET; all the latter showed fusion transcripts similar to those seen in tumors with the t(11;22) (q24;q12) translocation. The method is extremely useful in PNETs presenting in unusual sites, such as skin and kidney. Preliminary data in RMS have also shown validity of the method to detect alveolar RMS which most often shows t(2;13) (q35;q14) translocation. Unusual tumors with a mixed neural and mesenchymal phenotype (malignant ectomesenchymoma) can also be identified reliably. RT-PCR analysis should be added as an adjunct diagnostic technique in the diagnosis of small round cell tumors in children. In the UP section, it has been incorporated in the initial diagnostic work-up of these tumors.

The anti-MyoD1 antibody stained all RMS, even the poorly differentiated ones, and none of the nonmyogenous tumors. On the contrary, the 12E7 antibody stained intensely 99% of Ewing's sarcoma/PNET and none of the poorly differentiated RMS and NB. Occasionally, well differentiated rhabdomyoblastic cells were positive for 12E7. These tumors, however, do not constitute a cause for diagnostic dilemma in this group of tumors. Therefore, these two markers can be used in a complementary fashion in the diagnosis of this group of tumors.

Publications:

Roth M, Medeiros LJ, Kapur S, Wexler W, Mims S, Horowitz ME, Tsokos M. Malignant Schwannoma with melanocytic and neuroepithelial differentiation in an infant with congenital giant melanocytic nevus: A complex neurocristopathy. Hum Pathol 1993; 24:1371-5.

Tsokos M. The diagnosis and classification of childhood rhabdomyosarcoma. Sem Diagn Pathol 1994;11:26-38.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09172-06 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Senior Staff Fellow	LP NCI
	N. Guo	Visiting Associate	LP NCI
	J. Kaiser	IRTA Fellow	LP NCI

COOPERATING UNITS *(if any)*

H. Krutzsch, P. Browning, R.C. Gallo, P. Steeg, NCI; J. Inman, NIAID; D. Blake, Meharry Medical College, Nashville; J. Murphy-Ullrich, University of Alabama, Birmingham; T. Vogel, BioTechnology General, Rehovot, Israel

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.7	2.2	1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Our objective is to understand the role of the adhesive protein thrombospondin in regulating tumor cell adhesion, growth, motility, and metastasis. As is the case for many adhesive proteins, thrombospondin has binding sites for several matrix components and binds to several types of cell surface receptors. We have shown that thrombospondin promotes adhesion and migration of melanoma and several other tumor cell lines and modulates migration and proliferation of endothelial cells. We found that at least two domains of thrombospondin are required for these activities and that thrombospondin interacts with both protein and sulfated glycoconjugate receptors on cell membranes. Using synthetic peptides and recombinant fragments, we have identified three functional sites including a novel adhesive sequence in the type I repeats of thrombospondin that mimics the activities of the whole molecule for regulating cell adhesion, migration and proliferation. Recently, we have also identified specific sequences in thrombospondin that activate latent TGF-beta or inhibit its activation by intact thrombospondin. These peptides have potential clinical applications in cancer and other diseases associated with abnormal angiogenesis and in regulating wound repair, inflammatory responses, and fibrosis.

We are developing experimental approaches to understand the molecular mechanisms of these multiple interactions of cells with thrombospondin and the cellular responses they elicit. We are interested in the direct effects of thrombospondin expression on tumor cells, the role of thrombospondin in neovascularization of tumors, and its role in other tumor cell interactions with endothelium during metastasis.

Major Findings:

Thrombospondin is an inhibitor of angiogenesis that modulates endothelial cell adhesion, proliferation, and motility. Synthetic peptides from the second type I repeat of human thrombospondin containing the consensus sequence -Trp-Ser-Pro-Trp-and a recombinant heparin binding fragment from the amino-terminus of thrombospondin mimic several of the activities of the intact protein. The peptides and heparin-binding domain promote endothelial cell adhesion, inhibit endothelial cell chemotaxis to basic fibroblast growth factor (bFGF), and inhibit mitogenesis and proliferation of aortic and corneal endothelial cells. The peptides also inhibit heparin-dependent binding of bFGF to corneal endothelial cells. The antiproliferative activities of the peptides correlate with their ability to bind to heparin and to inhibit bFGF binding to heparin. Peptides containing amino acid substitutions that eliminate heparin-binding do not alter chemotaxis or proliferation of endothelial cells. Inhibition of proliferation by the peptide is time-dependent and reversible. Thus, the anti-proliferative activities of the thrombospondin peptides and recombinant heparin-binding domain result at least in part from competition with heparin-dependent growth factors for binding to endothelial cell proteoglycans. These results suggest that both the Trp-Ser-Xaa-Trp sequences in the type I repeats and the amino-terminal domain play roles in the anti-proliferative activity of thrombospondin.

CD4+ T cells were shown to attach on thrombospondin predominantly via the 70 kDa core region of the protein. Three receptors mediate adhesion on thrombospondin. An activation-independent receptor mediates adhesion of resting T cells. The $\alpha 4\beta 1$ (VLA-4) and $\alpha 5\beta 1$ (VLA-5) integrins mediate a rapid increase in adhesion following activation. The up-regulation of ß1 integrins is associated with the preferential adhesion of memory T cells on thrombospondin. Our current work demonstrates that ß1 integrins cooperate with sulfated glycolipids to mediate adhesion of small cell lung carcinoma (SCLC) cells to thrombospondin. However, antibodies to the $\alpha 4$ and $\alpha 5$ subunits do not inhibit SCLC adhesion and the cells fail to attach on fibronectin, suggesting that a novel a subunit may function as the thrombospondin receptor in these cells. The SCLC cells do not express the *THBS1* gene but express *THBS3*. In other studies, we have shown that loss of expression of *THBS1* in several paired cell lines is associated with tumor progression and increased metastatic potential.

Publications:

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 1993;121:469-77.

Yabkowitz R, Dixit VM, Guo N, Roberts DD, Shimizu Y. Activated T cell adhesion to thrombospondin mediated in part by the $\alpha 4\beta 1$ (VLA-4) and $\alpha 5\beta 1$ (VLA-5) integrins. J Immunol 1993;151:149-58.

Vogel T, Guo N, Krutzsch HC, Blake DA, Hartman J, Mendelevitz S, Panet A, Roberts DD. Modulation of endothelial cell proliferation, adhesion, and motility by recombinant heparin-binding domain and synthetic peptides from the type I repeats of thrombospondin. J Cell Biochem 1993;53:74-84.

Roberts DD, Cashel J, Guo N. Purification of thrombospondin from human platelets. J Tiss Cult Meth (in press)

Patents:

US Application 07/801,812. Heparin- and sulfatide-binding peptides from the type I repeats of thrombospondin. Allowed April 5, 1994. Continuation-in-part application filed March 21, 1994.

US Application 07/973,235. Peptide inhibitors of fibronectin and related collagen binding proteins. (pending)

Methods and compositions for stimulating and inhibiting TGFβ activity with regulatory peptides. filed May 3, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09173-06 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are studying the binding of fibronectin and recombinant and proteolytic fragments of this protein to *Candida albicans* to determine which of the numerus 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. Optimal conditions have been established to obtain high receptor expression. In contrast to the published literature, binding is not inhibited by Arg-Gly-Asp peptides but requires new sites of interaction between *C. albicans* and the heparin- and collagen-binding sites of fibronectin. A 30 kDa proteolytic fragment from the gelatin/collagen-binding domain of fibronectin is a potent inhibitor of fibronectin binding to *Candida albicans*, with a molar inhibition constant equal to that of intact fibronectin. We are using these binding assays to purify a novel receptor for fibronectin from *C. albicans*.

Major Findings:

A 30 kDa proteolytic fragment from the gelatin/collagen-binding domain of fibronectin is a potent inhibitor of fibronectin binding to *Candida albicans*, with a molar inhibition constant equal to that of intact fibronectin. Recombinant and proteolytic fragments from the cell-, the fibrin I-, and the heparin II-binding domains also inhibit fibronectin binding, but are 13 to 1000-fold less active. In suspension, binding of fibronectin to *C. albicans* is regulated by growth conditions and is specific, saturable, time dependent, reversible, and divalent cation-independent. Scatchard plot analyses indicate the presence of high affinity (K_d= 1.3 x 10^{-9} M) and low affinity (K_d= 1.2 x 10^{-7} M) receptors. Recombinant or proteolytic fragments from four binding domains of fibronectin promote adhesion of *C. albicans*. A recombinant fragment corresponding to the cell-binding domain but with the sequence Arg-Gly-Asp-Ser deleted promotes *C. albicans* adhesion and inhibits fibronectin binding to *C. albicans* with the same activity as the natural sequence. Furthermore, two peptides containing the Arg-Gly-Asp sequence, and the peptides CS-1 and Arg-Glu-Asp-Val did not block the binding of fibronectin to *C. albicans*. Thus, in contrast to the specific binding of soluble fibronectin, recognition of immobilized fibronectin by *C. albicans* is mediated by several domains of the protein. Interactions with the cell-binding domain are not mediated by the Arg-Gly-Asp or other known recognition sequences as it has been suggested. Binding of fibronectin also did not correlate with C_3d binding to the avirulent clones of *C. albicans* strain H_{12} or with iC_3b binding to variants of the strain 4918.

Publications:

Nègre E, Vogel T, Levanon A, Guy R, Walsh TJ, Roberts DD. The collagen binding domain of fibronectin contains a high affinity binding site for *Candida albicans*. J Biol Chem (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09174-06 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Role of 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:	J. Kaiser	IRTA Fellow	LP NCI

COOPERATING UNITS *(if any)*

H. Krutzsch, NCI; D. Blake, Tulane University, New Orleans, LA; D. Tyrell, Glycomed, Alameda, CA; T. Vogel, BioTechnology General, Rehovot, Israel

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.25	0.75	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.)*

Altered glycosylation of glycolipids, glycoproteins, or proteoglycans are frequently observed in tumor tissues and cultured cancer cell lines. These changes have diagnostic use but also can contribute to the ability of tumor cells to grow, induce neovascularization, metastasize, and avoid host immune surveillance. We are primarily interested in sulfated glycoconjugates and their interaction with extracellular matrix and cell surface adhesion molecules. These include thrombospondin, laminin, selectins, and apolipoprotein E. Human small cell lung carcinoma cell lines are used to examine expression and function of novel sulfated glycolipids. These glycolipids interact with thrombospondin and contribute to adhesion of SCLC cells on a thrombospondin matrix. Sulfated glycolipids on these cells are also being examined as potential ligands for selectin-mediated adhesion of SCLC cells to activated endothelium. Heparin oligosaccharides are being used to define the molecular basis for interactions of heparin and heparan sulfate proteoglycans with two binding motifs on the thrombospondin molecule.

Major Findings:

Recombinant human apolipoprotein E3 (apoE), purified from *E. coli*, inhibited the proliferation of several cell types, including endothelial cells and tumor cells in a dose- and time-dependent manner. ApoE inhibited both de novo DNA synthesis and proliferation as assessed by an increase in cell number. Maximal inhibition of cell growth by apoE was achieved under conditions where proliferation was dependent on heparin-binding growth factors. Thus, at low serum concentrations (0%-2.5%) basic fibroblast growth factor (bFGF) stimulated the proliferation of bovine aortic endothelial (BAE) cells several-fold. The bFGF-dependent proliferation was dramatically inhibited by apoE with an $IC_{50} \approx 50$ nM. Under conditions where cell proliferation was mainly serum-dependent, apoE also suppressed growth but required higher concentrations to be effective ($IC_{50} \approx 500$ nM). ApoE also inhibited growth of bovine corneal endothelial cells, human melanoma cells, and human breast carcinoma cells. The IC_{50} values obtained with these cells were generally 3 to 5 times higher than with BAE cells. Inhibition of cell proliferation by apoE was reversible and dependent on the time of apoE addition to the culture. In addition, apoE inhibited the chemotactic response of endothelial cells that were induced to migrate by a gradient of soluble bFGF. Inhibition of cell proliferation by apoE may be mediated both by competition for growth factor binding to proteoglycans and by an anti-adhesive activity of apoE. The present results demonstrate that apoE is a potent inhibitor of proliferation of several cell types and suggest that apoE may be effective in modulating angiogenesis, tumor cell growth, and metastasis.

Based on its inhibitory activities for endothelial cell proliferation and motility, we are examining the effect of apoE on Kaposi's sarcoma. ApoE blocked cell proliferation and chemotaxis of AIDS-KS derived spindle cells in response to activated lymphocyte conditioned medium and oncostatin M. ApoE also inhibited the formation of angiogenic lesions induced in Balb/c nude mice by AIDS-KS cells. These results suggest that apoE may be an effective therapeutic agent for treatment of Kaposi's sarcoma.

Bovine corneal endothelial cells showed a strong migratory response to specific simple sugars (D-glucose and sucrose, but not L-glucose, sorbitol, lactose, or D-galactose) at concentrations above 10 mM. Checkerboard analysis of the migratory responses in modified Boyden chambers indicated both chemotactic and chemokinetic effects. Serum starvation of the cultures increased the chemotaxis towards D-glucose and 2-deoxy-D-glucose, but not towards sucrose. Migration to sucrose and glucose were inhibited by chelation of extracellular calcium or by inhibition of Na+, K+ ATPase with ouabain. To date, this migratory response has been found only in corneal endothelial cells. Neither human melanoma cells, human breast carcinoma cells, bovine aortic endothelial cells, nor bovine microvascular endothelial cells migrated towards simple sugars, although all cell types migrated toward fibronectin in chemotaxis assays. After 16-19 passages in culture, bovine corneal endothelial cells retained their ability to migrate towards fibronectin, but lost their ability to migrate towards sugars. This loss of migratory response was accompanied by a 7-fold decrease in Na+, K+ ATPase activity. Although loss of Na+, K+ ATPase activity accompanied the loss of migratory response, pretreatment of cell cultures with 25 mM glucose did not stimulate, but rather lowered Na+, K+ ATPase activity in low or high passage cultures.

Publications:

Vogel T, Blake DA, Whikehart DR, Guo NH, Zabrenetzky VS, Roberts DD. Specific simple sugars promote chemotaxis and chemokinesis of corneal endothelial cells. J Cell Physiol 1993;157:359-66.

Roberts DD, Mecham RP. eds. Cell surface glycoconjugates: Structure and function. New York: Academic Press, 1993.

Vogel T, Guo N, Guy R, Drezlich N, Krutzsch HC, Blake DA, Panet A, Roberts DD. Apolipoprotein E: a potent inhibitor of endothelial and tumor cell proliferation. J Cell Biochem 1994;54:299-308.

Patents:

US Application 08/105,900. Method of inhibiting Kaposi's sarcoma. filed August 12, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00891-11 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	A. Arestad	Special Volunteer	LP NCI
	J. Murata	Visiting Fellow	LP NCI
	H. Lee	Visiting Fellow	LP NCI
	T. Clair	Chemist	LP NCI
	H. Krutzsch	Research Chemist	LP NCI

COOPERATING UNITS *(if any)*

R. Hopkins and P. Harley, PRI/Dyne Corp., FCRDC

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.4	4.2	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have 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 125,000 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. A partial cDNA clone of the protein was isolated by screening an A2058 cDNA expression library with the anti-peptide ATX-102 antibody. This sequence has been extended using oligonucleotides corresponding to known peptide sequences as primers in reverse transcription (RT) reactions followed by polymerase chain reaction (PCR) amplifications. At present, we have the putative protein sequence for ~95% of ATX. Database analysis of the known sequence has revealed a 45% amino acid identity and a 57% nucleotide identity with a human nucleotide pyrophosphatase and threonine kinase, PC-1, found on the surface of plasma cells. In addition, we are continuing to characterize the protein and its active site with respect to the role of glycan units and with respect to associated enzymatic activities.

Major Findings:

I. Anti-Peptide Antibodies

The 125,000 dalton ATX has been purified to homogeneity. Amino acid sequence has been obtained on 27 peptides, comprised of 297 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. Five of these purified antibodies, made against four different ATX peptides, were found to bind to a single 125 kDa protein on immunoblots. A sixth anti-peptide antibody, made against a peptide suspected of having two asparagine-linked carbohydrate chains (ATX-212), recognizes only the 105 kDa deglycosylated protein. To date, none of the anti-peptide antibodies recognizes the native ATX. However, one of the anti-peptide antibodies (anti-ATX-102) has been used for immunohistochemical staining of human breast cancer tissue. In these stains, the antibody appears to recognize tumor cells preferentially.

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 approached this problem by using a number of complementary approaches. Attempts to utilize degenerate oligos deduced from known peptide sequences were unsucessful, whether we used the oligos for screening cDNA libraries or for reverse transcription of mRNA followed by amplification with PCR. We then utilized the affinity-purified anti-peptide ATX-102 antibodies to screen an A2058 expression library prepared from size selected mRNA (>1000 bp) placed directionally into lgt11. Using this technique, we obtained a partial cDNA clone of ATX which we termed 4C11. This clone contained 1084 bp, including the polyadenylation signal site and the poly-A tail. It also includes a 628 bp open reading frame, encoding a 209 amino acid peptide. This clone has now been extended to nearly full length with sequence encoding 3020 bp which encodes a putative protein ~850 amino acids long. This putative protein sequence matches 26 previously identified ATX peptides. Northern blot analysis of poly-T-purified A2058 mRNA, indicates that the full-length cDNA clone will be ~3.3 kb long.

As a complementary project, we have begun to sequence the ATX gene from a human teratocarcinoma cell line (N-tera 2D1). We have used purified cDNA inserts from a previously prepared cDNA library as template for reverse transcription/ polymerase chain reaction amplification, using known A2058 sequence to synthesize oligonucleotide primers. In addition, we have succeeded in obtaining 5' ATX sequence from normal human liver mRNA using the 5' RACE kit of Clontech.

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 was bound by 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 several asparagine-linked carbohydrate chains.

We have now further characterized ATX as a glycoprotein. Several lectins bound to immobilized ATX, particularly concanavalin A (mannose) and galanthus nivalis agglutinin, both of which recognize mannose moieties. When ATX was treated with N-glycosidase F, which removes N-linked sugars at the asparagine residue, the molecular weight of ATX decreased to approximately 100-105 kDa, a loss of approximately 16% of the total molecular weight. Furthermore, when ATX was treated with trifluoromethane sulfonic acid which hydrolyses all sugar moieties, the molecular weight of ATX also decreased to approximately 105 kDa. In addition, treatment of ATX with N-glycosidase F, at concentrations sufficient to eliminate binding to concanavalin A, had no effect on its stimulation of cellular motility. In contrast, neither treatment with neuraminidase nor neuraminidase plus O-glycosidase significantly altered the molecular weight of ATX. These data are consistent with ATX having predominantly or exclusively N-linked carbohydrate moieties as side chains.

IV. Studies of ATX-associated Enzymatic Activities

Database analysis of ATX sequence revealed a 45% amino acid identity and a 57% nucleotide identity with a human nucleotide pyrophosphatase and threonine kinase, PC-1, found on the surface of plasma cells. Preliminary evidence has indicated that the isolated ATX protein exhibits similar enzymatic activities. For example, when (g)32P-ATP was incubated in the presence of semi-purified ATX, ATX became strongly labelled with 32P indicating possible autophosphorylation. Thin layer chromatography of the phosphorylated ATX (cut as a single band from a polyacrylamide gel) indicated that the phosphate group is exclusively linked to threonine.

In addition, ATX contains sequences homologous to bovine type I phosphodiesterase, the thrombospondin PAI-1 binding domain and the loop region of an EF hand which is a potential calcium-binding domain. ATX may thus define a new class of exoenzymes which could play a role in tumor cell motility.

Plans for Future Studies:

Our primary goal remains to complete the cloning of the 5′ end of the autotaxin gene. We currently have tentative 5′ sequence obtained from normal human liver mRNA. We plan to utilize this known sequence to synthesize oligonucleotides to be used as primers in reverse transcription, polymerase chain reaction amplifications. In addition, we will use commercially availble 5′ RACE kits to attempt to complete the cDNA sequence. 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. Large scale production of protein would also allow us to perform ATX active site analysis and to study the ATX cell surface receptor.

In addition, we are continuing to purify ATX from A2058 conditioned medium. We will use this purified protein for assays to test ATX-associated enzymatic activities. For example, we need to better define the kinase/phosphodiesterase activities of ATX. We can utilize non-hydrolyzable ATP analogs as competitive inhibitors, phosphatases to remove phosphate groups from ATX, and 32P-ATP to identify the specific threonine which is phosphorylated. We can also utilize enzyme substrates to study specificity and kinetics. In addition, it is important to determine wheher the enzymatic activities are important for the motility-stimulating activity of ATX.

Finally, the ATX anti-peptide antibodies will be used to perform more extensive 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. Further studies into the tumor specificity of ATX could utilize such techniques as microdissection with PCR amplification and *in vitro* hybridization.

Publications:

Stracke ML, Liotta LA, Schiffmann E. Protein factors that affect tumor cell motility. In: Jones G, Wigley C, Warn R, eds. Symposia of the Society for Experimental Biology, No. 47, Cell Behaviour: Adhesion and Motility. Cambridge, England: The Company of Biologists Limited, 1993;197-214.

Stracke ML, Murata J, Aznavoorian S, Liotta LA. The role of the extracellular matrix in tumor cell metastasis. In Vivo 1994;8:49-58.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09185-05 LP

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)*

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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:	PROFESSIONAL:	OTHER:
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

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

B

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

Directed migration of tumor cells is characterized by cycles of pseudopod protrusion and attachment at the leading edge of the cell, contraction of the cell body, and detachment of the trailing end. This coordinated series of events is mediated by specific receptors and intracellular signaling pathways. In the course of antibody inhibition studies to identify the type IV collagen motility receptor(s), we discovered that A2058 cells express the integrin receptor for vitronectin (Vn), $\alpha v\beta 3$. Further examination of vitronectin-mediated chemotaxis (CTX) and haptotaxis (HTX) revealed similarities and differences relative to type IV collagen-induced motility. Specifically, Vn-mediated CTX is inhibited 70-80% by pertussis (PT), although not as completely as is type IV-CTX (>90%), indicating involvement of a similar type of G protein in transduction of signals from both ligands. Blocking antibodies to integrin $\alpha v\beta 3$ inhibited Vn-mediated, but not type IV-mediated, migration; $\alpha v\beta 3$ integrin is therefore indirectly linked to G protein activation in Vn-CTX. Peptides containing the RGD sequence abolished Vn-mediated, but not type IV-mediated migration, further illustrating that distinct receptors are involved for each ligand. HTX to Vn was only slightly (<20%) inhibited by PT; therefore, integrin $\alpha v\beta 3$ transduces motility signals from soluble Vn largely through a G protein, whereas signaling from insoluble Vn involves multiple pathways, with G proteins playing a minor role. Preliminary studies have shown that insoluble Vn stimulates tyrosine phosphorylation of certain proteins, and that inhibitors of tyrosine kinases inhibit Vn HTX. Further study of this defined ligand-receptor system should yield valuable information about integrin-mediated signals involved in tumor cell pseudopod protrusion and migration. Comparison with type IV collagen-induced migration will likely reveal some generalities of matrix-mediated motility, as well as certain distinctions.

Major Findings:

Active tumor cell locomotion is a component of several stages of the metastatic cascade, including initial dissemination of cells from the primary tumor, invasion through interstitial matrix, and intravasation/extravasation of blood vessels. Extracellular matrix (ECM) components, both insoluble and as partially degraded soluble fragments, are likely to play a prominent role in stimulating and promoting migration of tumor cells. Integrin receptors for ECM components mediate many morphological and functional changes of cells including those involved in the motility response; however, the intracellular signals transduced through integrins are largely unknown and are only beginning to be actively investigated. Type IV collagen stimulates migration of A2058 human melanoma cells through an as yet unknown receptor. Migration to the soluble ligand is transduced through a pertussis toxin (PT)-sensitive G protein. In the course of antibody inhibition studies to identify the receptor(s) mediating type IV collagen-induced migration, we discoverd that A2058 cells express abundant $\alpha v\beta 3$, an integrin receptor for vitronectin (Vn), and much lower levels of another Vn receptor (VnR), $\alpha v\beta 5$. Immunofluorescent staining of cultured cells showed that $\alpha v\beta 3$ localized to focal adhesions, and that $\alpha v\beta 5$ was diffusely distributed over the cell surface. Further examination of the effects of Vn on A2058 cells showed that Vn is a potent motility-inducing agent, both soluble (chemotaxis, CTX) and insoluble (haptotaxis, HTX). PT treatment of cells inhibited Vn-CTX by ~70-90%, similar to previous results with type IV collagen. However, the effect of PT on Vn-HTX was minimal (<20%). Blocking antibodies to the integrin $\alpha v\beta 3$ completely inhibited CTX to Vn, while HTX to Vn was inhibited by 40-60%; either an additional, unidentified VnR is involved in HTX, or else antibody inhibition is less effective in this type of assay. (Antibodies to the $\beta 1$ integrin subunit, and to $\alpha v\beta 5$, were without effect). Pretreatment of cells with peptides containing the RGD sequence completely inhibited Vn CTX and HTX, indicating an absolute requirement for interaction of VnR(s) with the RGD sequence in Vn. In contrast, anti-$\alpha v\beta 3$ did not inhibit type IV collagen-induced motility, nor did RGD-containing peptides. Therefore, although Vn and type IV collagen share a similar signal transduction mechanism, Vn-stimulated motility is primarily mediated by $\alpha v\beta 3$, whereas type IV collagen utilizes a distinct receptor(s). Preliminary experiments were performed to examine the role of tyrosine phosphorylation in Vn-mediated motility. These indicated that substratum-bound Vn stimulated tyrosine phosphorylation of proteins ~68-70 kDa, in contrast to soluble Vn over a similar time course (using concentrations of Vn which maximally stimulate motility). Also, genistein, a tyrosine kinase inhibitor, partially inhibited Vn-mediated HTX (results with CTX are not yet established). With Vn and its receptor $\alpha v\beta 3$, we now have a defined ligand-receptor system with which to study the biochemical mechanisms of matrix-mediated CTX and HTX, and to further differentiate the signaling pathways for these two types of motility responses. Future experiments will aim to identify the proteins which are differentially tyrosine-phosphorylated in cells exposed to insoluble vs. soluble Vn, and to compare these results with those of similar experiments with type IV collagen. To extend these studies, tyrosine-phosphorylated proteins in cells which have migrated through a filter will be compared with those of non-migrating cells. Pseudopod preparations from cells which have migrated in response to a variety of

ligands will be examined for phosphotyrosines; preliminary Western immunoblots have revealed phosphotyrosine in pseudopods elicited by type IV collagen. It is very likely that proteins present in pseudopods of stimulated cells are involved in effecting migration, and these will continue to be resolved and identified by 2-D gel electrophoresis and Western immunoblotting. Also, the mechanism of G protein coupling to integrin $\alpha v\beta 3$ will be examined.

Other signaling pathways could contribute to motility and involve serine/threonine kinases, calcium-regulated functions, arachidonic acid metabolism, and isoprenoid metabolism. Specific inhibitors and stimulators of these pathways are available and will be tested for their effect in CTX and HTX. With our collaborator, Dr. Cheng Dong of Pennsylvania State University, we will continue to examine the effects of these agents on the dynamics of protrusion of individual pseudopods. Approximately 80% of effort will therefore be involved in the elucidation of chemotactic and haptotactic signals elicited by Vn, type IV collagen, and other ligands as time allows. Results will be integrated with our previous work with isolated pseudopod preparations, as we continue to define the signals and proteins that differentiate migrating from non-migrating cells.

Cloning and sequencing of the PT-sensitive G proteins in A2058 cells will be resumed, with the aim of identifying which G proteins are involved in Vn- and type IV collagen-mediated CTX. Also, RT-PCR and differential display and/or subtractive hybridization will be attempted on isolated pseudopod preparations and on a selected population of highly migratory cells to identify genes differentially expressed in migratory vs. non-migratory cells. This will probably comprise ~20% of effort.

Publications:

Dong G, Aznavoorian S, Liotta L. Two phases of pseudopod protrusion in tumor cells revealed by a micropipette. Microvas Res 1994;47:55-67.

Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Ann Rev Cell Biol 1993;9:541-73.

Stracke ML, Murate J, Aznavoorian S, Liotta L. The role of the extracellular matrix in tumor cell metastasis. In Vivo 1994;8:49-58.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09352-04 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

AAMP-1, a 57 kDa Protein Newly Identified in Human Tissues

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

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1.25	1.00	0.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The cDNA that encodes AAMP was used to produce a fusion recombinant protein. A significant quantity of the thrombin digested form of recombinant AAMP has been purified lacking its fusion partner (glutathinoe-S-transferase). It has been used to stimulate polyclonal antibodies that are then affinity-purified. It has also been trypsin digested to provide 24 peptides for amino acid sequencing. Large segments of the DNA coding message for AAMP have been verified by these sequencing results. These regions of confirmed sequence include the predicted heparin binding domain, immunoglobulin type domains, and a single predicted transmembrane region. Reactivity of affinity-purified anti-recombinant AAMP with the 57 kDa protein that also reacts with anti-peptide 189 (AAMP derived peptide) in human tissues confirms the identity of this protein as AAMP. Both antibodies react with the same protein that is present in many tissues and increases with activation of T cells standardized either according to cell number or amount of protein. Anti-recombinant AAMP reacts with the same protein in all human tissues tested (heart, lung, liver, skin, lymph node, skeletal muscle, kidney, brain, and metastatic melanoma). This widespread distribution corresponds to what was seen in RNA blots of AAMP's 1.6 kb message. Other species that contain either AAMP protein or RNA message include rat and cow at this point. A shared six amino acid epitope between AAMP's primary sequence and alpha-actinin (conformational epitope only) in all tissues has been identified by reactivity with anti-P189. This same small epitope is also shared with a 21 kDa protein from skeletal muscle that appears to be restricted to fast fibers as indicated by immunoperoxidase staining. Functional studies with P189 include heparin binding (ongoing) and cell motility. P189 (200 µg/ml) inhibits A2058 cell chemotaxis 24%, compared to a similar peptide lacking its heparin binding domain. The difference is significant ($p < .0001$).

Future Plans:

1. The chromosomal localization of AAMP and its genomic sequence will be studied. Hopefully information regarding its regulation can be obtained.

2. Additional DNA and peptide sequencing of AAMP will be used to determine its amino terminus and to confirm sequence that has not been confirmed yet.

3. AAMP recombinant protein with an intact heparin binding domain needs to be purified for functional studies in tumor cells, T lymphocytes, and other benign cells (heparin/cell binding, extracellular matrix adhesion, motility inhibition/stimulation, etc.). Thrombin digestion which is necessary for current purification methodology partially degrades this domain.

4. Anti-AAMP recombinant protein antibody is being used to study the distribution of AAMP in tissues, its cellular localization, and expression of AAMP in samples of human tissue from malignancies compared with benign counterparts.

5. Information about AAMP's function will be obtained with antisense methods.

6. The diagnostic usefulness of AAMP in distinguishing reactive from malignant cells will be determined.

7. The effects of AAMP expression on T cells, macrophages, and stromal cells and on their interactions with tumors and benign counterparts (when available) will be determined.

8. AAMP protein from mammalian tissue needs to be purified for further confirmation of its sequence, determination of the amino terminus, and detection of posttranslational modifications. The 57 kDa protein in brain tissue (also in calf brain) will be the candidate protein for purification.

9. The peptide, P189, derived from AAMP's amino terminal region, that binds either cells or heparin will be further evaluated for its medical usefulness as a heparin binding agent. Its dissociation constant for heparin binding in its solubilized and aggregated forms will be determined.

10. Anti-P189's specific staining of fast skeletal muscle fibers and the significance of its reactivity with alpha-actinin will be further evaluated through collaboration with others who study muscle biology.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00892-11 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Molecular Biology of the Metastatic Phenotype

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

PI:	P. Steeg	Chief, Women's Cancers Section	LP NCI
OTHER:	N. MacDonald	Visiting Fellow	LP NCI
	U. Flatow	Biologist	LP NCI
	A. De La Rosa	Visiting Fellow	LP NCI
	J. Freije	Guest Researcher	LP NCI
	R. Manrow	Expert	LP NCI

COOPERATING UNITS (if any)

Dr. M. Bissel, U. Cal., Berkeley, Dr. M. Grever, DTP, DCT, NCI; Dr. R. Callahan, DCBDC, NCI; Dr. M. Merino, Chief, Surgical Pathology Section, LP, DCBDC, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Women's Cancers Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS	PROFESSIONAL	OTHER
3.8	3.3	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.)

Basic and translational research has investigated the role of *nm23* in the regulation of tumor metastasis. Reduced *nm23* expression has been correlated with poor clinical course in many tumor cohorts, and transfection data in breast carcinoma and melanoma cell lines indicate that over-expression of *nm23* can reduce in vivo metastatic potential by 50-90%.

The biochemical mechanism of Nm23 suppression of metastatic potential is under investigation. We have discovered a serine phosphorylation of Nm23 which is correlated with its biological suppressive capacity. Site directed mutagenesis of the *nm23* cDNA is underway to directly test this association. Yeast two-hybrid cloning is being performed to identify proteins that specifically interact with Nm23.

The molecular regulation of *nm23* expression is also under investigation. Cohort analysis indicates that reduced *nm23* expression found in aggressive breast carcinomas is likely due to transcriptional regulation. The *nm23-H1* promoter has been cloned, and is under analysis. Interestingly, a series of motifs previously reported to confer mammary-specific gene regulation has been found in the *nm23-H1* promoter.

Translational research has identified 45 novel pharmaceutical agents which are selectively inhibitory in vitro to low *nm23* expressing (i.e., aggressive) human breast carcinoma and melanoma cell lines. One of these agents stimulated the Nm23 expression of a metastatic human breast carcinoma cell line. Continued in vitro characterization of the effects of each agent on Nm23 biochemistry and metastasis biology is underway in anticipation of in vivo animal experimentation.

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 murine melanoma cell lines. To date, histopathological and/or clinical course correlates of metastatic disease have been associated with reduced *nm23* expression in cohorts of breast, hepatocellular, gastric, ovarian, and cervical carcinomas as well as melanoma. Mutation of *nm23*, rather than its reduced expression, has been identified in metastatic colorectal carcinoma and neuroblastoma lesions. Transfection of murine *nm23-1* cDNA into a murine melanoma cell line, and human *nm23-H1* cDNA into a human breast carcinoma cell line reduced their in vivo metastatic potential by 50-90%. An independent transfection of the Drosophila homolog of *nm23* into flies expressing the *tum-1* oncogene, also increased viability. In vitro correlates of reduced in vivo metastatic potential in *nm23* transfected cell lines include reduced motility and colonization responses to cytokines, as well as morphological, biosynthetic and polarization evidence of duct differentiation in the breast carcinoma cells. The data suggest that *nm23*, in some tumor cell types, may function as a metastasis suppressor gene.

The biochemical mechanism of Nm23 suppression of metastasis remains a major area of investigation in my laboratory. Nm23 proteins possess a nonspecific nucleoside diphosphate kinase (NDPK) activity. We have recently described a serine phosphorylation of Nm23, which is thermodynamically independent of its histidine phosphorylation in the NDPK activity. Phosphorylation of serines 44 and possibly 120, 122 and 125 of Nm23 were found. Mass spectroscopy evaluation of Nm23 phosphorylation is ongoing, to confirm and extend these data. Serine phosphorylation was found to be inhibitable by cAMP in vitro, and forskolin in vivo. The biological significance of Nm23 serine phosphorylation was suggested by its correlation with Nm23 expression levels and in vivo metastatic potential among control- and *nm23* transfected melanoma and cell lines, in contrast to NDPK data. We have extended this correlation to control- and *nm23-H1* transfected human breast carcinoma cell lines. To identify proteins which specifically interact with Nm23 on its functional biochemical pathway, we are utilizing the yeast two-hybrid system. The Nm23-H1 coding region has been subcloned into an expression plasmid adjacent to the DNA binding regions of the yeast transcription factor GAL4. Interaction of the Nm23-H1 fusion protein with a product of a cDNA library that expresses proteins fused to the GAL4 transcriptional activation domain results in the stimulation of the transcription of HIS3 and β-galactosidase reporter genes downstream of a GAL4 binding site. The Nm23-H1-GAL4 binding domain has been introduced into the appropriate strain of yeast, and we plan to start screening a cDNA library for protein:protein interactions in the near future.

To correlate Nm23 biochemistry with its biological effects, we are performing site directed mutagenesis of the *nm23-H1* cDNA. To date, we have altered amino acids coding for the histidine involved in its NDPK activity, serines 44 and 120, as well as every mutation reported in human tumors and aberrant Drosophila development. The wild-type and site-directed mutant cDNAs have been cloned into a eukaryotic expression vector as well as a bacterial expression vector, and their sequences confirmed. We plan to transfect each construct into a human

breast carcinoma cell line to determine the effect of its expression on metastatic potential in vivo, and characterize the biochemistry of each mutant protein when produced and purified from bacteria. The data may identify which biochemical features of Nm23 are critical to its biological effects.

Molecular analysis of the regulation of *nm23* expression is also underway. We have asked how *nm23* expression levels are reduced in highly aggressive breast carcinomas. In collaboration with Dr. Robert Callahan, we simultaneously determined *nm23-H1* allelic deletion, *nm23* coding region mutations, Nm23-H1 protein expression and patient metastasis free survival in an infiltrating ductal carcinoma cohort. The data indicated that, while allelic deletion of *nm23-H1* occurs, it does not uniformly correlate with reduced Nm23-H1 protein expression or patient clinical course. Low Nm23-H1 protein levels significantly correlated with poor patient survival, and are thought to be regulated by transcriptional mechanisms. We have identified and cloned 2 kb of the 5' promoter region of the *nm23-H1* gene. Within the promoter region there are two putative AP1 binding sites and a putative CTF/NF1 binding site. The promoter has no obvious TATA box, but has a consensus Spi-1/PU.1 site located in the vicinity of the putative transcriptional start site. Spi-1/PU.1 are members of the ETS gene family of transcription factors. PU.1 has been shown to interact with TFIID, thus possibly serving the same function as the TATA box in TATA-less promoters. Furthermore, the *nm23-H1* promoter sequence exhibits a strong degree of homology to a series of binding sites known to be involved in mammary cell specific gene expression, including the CTF/NF1, an ACAAAG recognition site, and the ETS-like consensus sequence G/C A/C G G A A/T G T/C (which is G A G G A A G C in *nm23-H1*). Further characterization of this potentially interesting set of motifs may shed light on the regulation of *nm23-H1* expression in breast carcinoma cells. Once characterized, we plan to determine whether *nm23-H1* transcription is also regulated by mutations in its promoter region in a tumor cohort.

Translational research has been initiated to identify pharmaceutical agents which are selectively active (cytotoxic/cytostatic) against low *nm23* expressing tumor cells, in collaboration with the Developmental Therapeutics Program, DCT, NCI. A panel of 14 human breast carcinoma or melanoma cell lines were assayed for Nm23 protein expression levels by densitometry of Western blots; these data were correlated with tumor cell line sensitivity to various agents using the COMPARE program. When tumor cell line Nm23 expression was correlated to sensitivity to standard agents, i.e., those agents currently used in practice or in clinical trial, none were identified with a correlation coefficient of > -0.70 that were selectively active against low *nm23* expressing, aggressive tumor cell lines. This correlation reinforces our understanding that we need to develop new agents which are active against the most aggressive tumor cells. A similar correlation of tumor cell *nm23* expression and sensitivity to pharmaceutical agents was run using a base of 30,000 novel agents. Approximately 45 agents were identified with correlation coefficients ranging from -0.70 to -0.90 of inhibitory activity against low *nm23* expressing tumor cells.

Fourteen of these agents have been examined in preliminary in vitro experiments in my laboratory to date. Of the fourteen, we have verified that seven are significantly more inhibitory to the low *nm23* expressing, metastatic MDA-MB-435 breast carcinoma cell line than to the unrelated, high *nm23* expressing,

nonmetastatic MCF-7 breast carcinoma cell line. Of the seven, one agent stimulated the *nm23* expression of MDA-MB-435 breast carcinoma cells, when an ID50 dose was incubated with cells for 2 days. Another agent did not directly stimulate *nm23* expression of MDA-MB-435 tumor cells; however, when its inhibitory activity was compared on control- and *nm23-H1* transfected MDA-MB-435 tumor cell clones, it exhibited significantly less inhibitory activity against the *nm23-H1* transfectant. This agent may exert its inhibitory effect at a position downstream of *nm23* on its functional biochemical pathway. The remaining five agents are assumed at this point to operate independently of the *nm23* pathway. All seven agents inhibited bovine aortic endothelial cell growth, suggesting an angiogenesis-inhibitory function. Additional assays of *nm23* biochemistry, as well as aspects of metastatic potential are planned for all seven agents, in anticipation of in vivo animal testing for anti-growth and anti-metastatic function.

In agreement with the COMPARE analysis of *nm23* expression and sensitivity to standard agents, we have observed that high *nm23* expressing human breast, ovarian and prostatic carcinoma cell lines, as well as murine melanoma lines, obtained by transfection, are more sensitive to growth inhibition in response to the alkylating agent cisplatin. Using control- and *nm23-1* transfected murine melanoma cell lines, we have demonstrated that the *nm23-1* transfectants are significantly more sensitive to cisplatin inhibition of lung colonization in vivo. The mechanism of *nm23* modulation of tumor cell cisplatin sensitivity apparently does not entail induction of apoptosis, *mdr* expression levels, glutathione levels, or rate of double-stranded DNA repair. Using human breast carcinoma cell lines, we have observed an increase in the formation of cisplatin induced interstrand cross links in *nm23-H1* transfectants. The data suggest the potential use of agents which elevate Nm23 expression, if and when they are developed from the DTP screening process, in combination with standard alkylation agent therapy.

Publications:

MacDonald NJ, Steeg PS. Molecular basis of tumor metastasis. Cancer Surveys 1993;16:175-99.

Steeg PS, De La Rosa A, Flatow U, MacDonald NJ, Benedict M, Leone A. Nm23 and breast cancer metastasis. Breast Cancer Res Treat 1993;25:175-87.

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 1993;8:2325-33.

Kantor JD, McCormick B, Steeg PS, Zetter BR. Inhibition of cell motility after nm23 transfection of human and murine tumor cells. Cancer Res 1993;53:1971-3.

Weinstat-Saslow D, Steeg PS. Angiogenesis and colonization in the tumor metastatic process: Basic and applied advances. FASEB J (in press)

MacDonald NJ, De La Rosa A, Benedict MA, Freije JMP, Krutzsch H, Steeg PS. A serine phosphorylation of nm23, and not its nucleoside diphosphate kinase activity, correlates with suppression of tumor metastatic potential. J Biol Chem 1993;268:25780-9.

Cropp CS, Lidereau R, Leone A, Liscia D, Cappa APM, Campbell G, Barker E, LeDoussal V, Steeg PS,, Callahan RS. NME1 expression and loss of heterozygosity mutations in primary human breast tumors. J Natl Cancer Inst (in press)

De La Rosa A, Williams RL, Steeg RS. Nm23/nucleoside diphosphate kinases: Toward a structural and biochemical understanding of its biological functions. Bioessays (in press)

Bertheau P, De La Rosa, A., Steeg PS, Merino MJ. Distribution of Nm23 protein in neoplastic and non-neoplastic thyroid tissues. Am J Pathol (in press)

Patents:

U.S. Patent 5,049,662 - kit for diagnosing cancer metastatic potential

Continuation-in-Part 528,713 - therapy of cancer using nm23

Continuation-in-Part 775,081 - modulation of tumor cell sensitivity to alkylating agents

U.S. Patent Application 422,801 - prognostic use of human nm23 genes

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09377-02 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Women's Cancers Section	LP NCI
OTHER:	D. Weinstat-Saslow	Staff Fellow	LP NCI
	R. Manrow	Expert	LP NCI
	J. Freije	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

Maria Merino, Chief, Surgical Pathology Section, Laboratory of Pathology, DCBDC, NCI; David Page, Vanderbilt University School of Medicine

LAB/BRANCH

Laboratory of Pathology

SECTION

Women's Cancers Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS	PROFESSIONAL	OTHER:
1.6	1.6	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

While molecular alterations in infiltrating ductal breast carcinomas are relatively well characterized, little is known about the molecular changes in early breast disease. We are in the process of determining the mRNA expression patterns of cell cycle associated and DNA repair genes in a cohort of human preneoplastic and carcinoma-in-situ (CIS) lesions. To date, cyclin A mRNA levels have been shown to be expressed in a uniform pattern in most breast lesions, as opposed to its cell cycle regulated pattern in many normal tissues. Levels of cyclin A mRNA were highest in the more advanced lesions, including atypical ductal hyperplasia, cribiform noncomedo DCIS and comedo DCIS. This trend suggests a causal role of overexpressed and deregulated cell cycle associated cyclin in the initial stages of breast neoplasia, a hypothesis to be tested in transfection experiments. Similar experiments are in progress for cyclin D1, and the DNA repair-related genes ERCC-1, ERCC-2, ERCC-3, ERCC-5, ERCC-6, DNA polymerase β, DNA ligase I, hMSH2, p53, and WAF1. In addition, the contribution of telomerase to early breast progression is under investigation, through examination of its activity at various stages of progression, and attempts to clone the telomerase gene and its intrinsic RNA. These experiments are expected to contribute to a molecular definition of breast preneoplasia, which may lead to preventative approaches.

Major Findings:

While molecular alterations in infiltrating ductal carcinoma cells of the human breast are well characterized, relatively little is known about alterations in preneoplastic and carcinoma-in-situ (CIS) lesions. These lesions signal a significant increase in patient risk for the subsequent development of invasive breast carcinoma and, in some cases, may be precursor lesions. The reasons for the relatively poor molecular characterization of human premalignant and CIS lesions may include their small size, as well as the lack of cell lines for study. A molecular characterization of these lesions may improve prognostication for affected women, and may point to novel chemopreventative approaches.

We have investigated the expression of several cell cycle related genes in a cohort of premalignant and CIS lesions, using in situ hybridizations on sections of formalin fixed, paraffin embedded material. Probes complimentary to cyclin A mRNA, normally expressed at the G2-M boundary of the cell cycle, and cyclin D1 mRNA, normally expressed at the G1-S boundary of the cell cycle, have been used to date. Both cyclins have recently been reported to be overexpressed in a percentage of human breast tumors, leading to the hypothesis that they may be oncogenes. For cyclin A, approximately 37 lesions have been hybridized with the probe and evaluated for grain density (mRNA expression level) and histology by a pathologist and two molecular biologists. Grain densities were evaluated on a 1-5+ basis, with 1 representing the lowest, and 5 representing the highest grain density. To date, relatively uniform levels of cyclin A expression have been noted among cells within each lesion. These data suggest that transcriptional control of cyclin A expression in these lesions is not cell cycle regulated, unlike many normal tissues. While fairly uniform between the cells of a section, overall grain densities (cyclin A mRNA levels) varied between lesions, from 1+ to 5+. High cyclin A expression levels (5+) may be more apparent in the more advanced lesions. High cyclin A levels were observed in 20% (1/5) typical hyperplasias, 0% (0/8) atypical ductal hyperplasias, 11% (1/9) noncomedo DCIS, 33% (1/3) papillary noncomedo DCIS, 0% (0/2) atypical lobular hyperplasias, 67% (4/6) cribiform noncomedo DCIS, and 75% (3/4) comedo DCIS. In atypical ductal hyperplasia, cyclin A expression was concentrated just below 5+, at the 4+ level, in 63% (5/8) lesions. An additional 23 lesions have been hybridized with the cyclin A probe and are awaiting review, while ~30 lesions remain to be hybridized to complete this phase of the study. The histology and grain density of each lesion will be independently evaluated by David Page, M.D., Vanderbilt University. The data, while preliminary, suggest that elevation of cyclin A mRNA levels correlates with aggressive histology in preneoplastic and CIS lesions.

In a similar manner, cyclin D probes have been hybridized to 60 lesions, and an additional ~30 lesions await hybridization and evaluation. To date, uniform expression of cyclin D1 has also been observed in preneoplastic and CIS lesions, in contrast with that found in many normal tissues. Where tested, cyclins A and D1 levels, on a 1+ to 5+ basis, have not uniformly covaried, indicating their independent transcriptional regulation. Transfection experiments are planned to determine whether changes in cyclin expression has a correlative or cause-and-effect relationship with breast neoplastic progression.

A second focus of molecular investigation in preneoplastic and CIS breast lesions concerns their cellular levels of DNA repair. Loss of DNA repair capability and cell cycle control in response to DNA damage have been postulated to play a role in both carcinogenesis and malignant progression. In situ hybridization experiments are planned to determine the mRNA levels of the ERCC-1, ERCC-2, ERCC-3, ERCC-5, ERCC-6, DNA polymerase-β, DNA ligase I, hMSH2 mismatch repair, p53, and WAF-1 genes. At present, each of these genes has been cloned into a riboprobe vector and confirmed by restriction analysis and DNA sequencing. We are assessing probe specificity and sample hybridization and washing conditions.

The enzyme telomerase is a reverse transcriptase bearing an intrinsic template RNA, responsible for adding the TTAGGG telomeric repeats found at the ends of chromosomes which are required to maintain their integrity. Somatic cells do not normally exhibit telomerase activity, and it has been suggested that the absence of this activity safeguards against uncontrolled cell proliferation. At some point in cancer development/progression telomerase expression becomes reactivated, possibly contributing to the immortal phenotype. The role of telomerase in breast neoplasia will be examined. We are attempting to purify this enzyme and/or its template RNA from human cells to acquire specific probes. Thus far, we have confirmed the existence of active telomerase in extracts of HeLa cells, and we have partially purified the enzyme by DEAE-Sepharose and gel filtration chromatography. We also plan to attempt the cloning of a portion of the intrinsic RNA sequence using 5' RACE technology and degenerate oligomers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09378-02 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Biological Consequences of Altered Gene Expression

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

PI:	P. Steeg	Chief, Women's Cancers Section	LP NCI
OTHER:	D. Weinstat-Saslow	Staff Fellow	LP NCI

COOPERATING UNITS *(if any)*

D. Slamon, UCLA; V. Zabrenetzky, Sr. Staff Fellow, Lab. of Path., DCBDC, NCI; D. Roberts, Chief, Biochemical Pathology Section, Lab. of Path., DCBDC, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Women's Cancers Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This report summarizes several projects requiring relatively minor levels of effort in the Women's Cancers Section, LP. Transfection of two cDNAs into the metastatic human breast carcinoma cell line MDA-MB-435 has been initiated, to determine the effects of altered gene expression on breast cancer tumorigenesis and metastatic potential. Transfection of cDNA encoding the extracellular matrix component thrombospondin resulted in reduced primary tumor size and metastatic potential in vivo. An angiostatic function is proposed, as culture supernatants of the thrombospondin transfectants inhibited the movement of endothelial cells in vitro, and primary tumors formed by thrombospondin transfectants contained reduced microvessel densities in vivo. A transfectant lacking the wild-type C-terminus of thrombospondin did not exhibit reduced primary tumor size or metastatic potential, suggesting a novel region of this protein to be biologically active.

Transfection of the c-*erb-B-2* oncogene into MDA-MB-435 breast carcinoma cells was without significant effects on primary tumor size, but increased pulmonary metastatic potential by 2-4-fold. These data may contribute to the explanation of why patients with c-*erb-B-2* overexpressing breast and ovarian tumors have a poor clinical course.

Major Findings:

This report summarizes several projects requiring relatively minor amounts of effort in the Women's Cancers Section, LP. Transfection of two cDNAs into metastatic human MDA-MB-435 breast carcinoma cells has been initiated, to determine the effect of gene expression on breast tumor formation and metastatic potential. This model system was first developed for *nm23-H1* transfection experiments, where *nm23-H1* overexpression resulted in a 50-90% inhibition of tumor metastatic potential in vivo. Ancillary studies with transfected MDA-MB-435 cell lines have included analysis of their in vitro motility, and morphological, biosynthetic and polarization analysis of differentiation in vitro.

Reduced expression of the extracellular matrix component thrombospondin was observed in aggressive murine melanoma, human breast carcinoma and human lung carcinoma cell lines, suggestive of an inhibitory function. To test this hypothesis, we have transfected the full length thrombospondin (THBS-1) cDNA into MDA-MB-435 breast carcinoma cells. Two transfectants expressing increased wild-type THBS-1, one transfectant expressing a C-terminus truncated THBS-1, and two control-transfectants have been investigated. Expression of THBS-1 by MDA-MB-435 cells resulted in a reduction in primary tumor size upon injection into the mammary fat pad of nude mice. The extent of primary tumor inhibition directly correlated with the amount of THBS-1 overexpressed. Formation of pulmonary metastases by the THBS-1 transfectants was approximately 50% of control transfectants. In each of these assays, the C-terminally truncated THBS-1 transfectant exhibited a phenotype comparable to that of the control transfectants, suggesting that previously unrecognized sequences in its C-terminus may be biologically relevant.

In vitro characterization of the control-, THBS-1 transfected and C-terminally truncated THBS-1 transfected cells is ongoing. No differences in tumor cell growth rate on plastic or colonization in soft agar have been observed. Culture supernatants from THBS-1 transfectants were significantly more inhibitory for the chemokinesis of endothelial cells, suggesting that thrombospondin is exerting an angiogenesis-inhibitory function. Microvessel densities have been determined in sections of primary tumors from each of the transfected cell lines, and the lowest densities are most often found in the THBS-1 transfected primary tumors, consistent with this hypothesis. The data suggest that increased thrombospondin expression by tumor cells may inhibit tumor angiogenesis, with resultant effects on both primary tumor formation and metastatic potential. Site directed mutagenesis is planned to identify the region of the thrombospondin protein in its C-terminus responsible for these effects, with the ultimate goal of testing the effect of this peptide in vivo.

In a similar fashion, the c-*erb-B-2* (*Her-2/neu*) oncogene has been transfected into MDA-MB-435 breast carcinoma cells. Overexpression of c-*erb-B-2* has been widely reported to correlate with poor prognosis in breast and ovarian carcinoma patients, but conflicting data have been reported on its effects on tumor growth. Transfection of c-*erb-B-2* at levels found in human tumors resulted in no significant increase in primary tumor size, but a doubling of pulmonary

metastatic potential. An even more impressive effect on metastatic potential was noted when the number of pulmonary lesions was determined, as opposed to the percentage of animals with metastases. Mice injected with c-*erb-B-2* transfectants were four-times more likely to develop lungs filled with metastases (>10 lesions in at least two different lobes) as control transfectants. The data indicate that *erb-B-2* expression impacts tumor metastatic potential, which may explain the reduced survival of these patients. In vitro characterization of these lines is ongoing, as is determination of the effects of Heregulin (the erb-B-2 ligand) on tumor biology.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09164-07 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Extracellular Matrix Pathology Section	LP NCI
OTHER:	D. Kleiner	Expert	LP NCI
	O. Malykh	Special Fellow	LP NCI
	M. Buck	Clinical Associate	LP NCI
	B. Birkedal-Hansen	NRC Associate	LP NCI
	R. Hewitt	Fogarty Fellow	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Extracellular Matrix Pathology Section

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In 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 TGFß1, 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.

A major obstacle to research in this area has been to localize MMP-2 activity within human tumor tissues. We have developed a method for quantitating the level of MMP-2 and its state of activation following microdissection of frozen tumor tissue. Using this method, we have localized only active MMP-2 species to the invasive front in human colorectal and breast cancer samples.

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 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.

2. Synthetic peptides containing the highly conserved region from the amino terminal profragment of the 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*.

3. The cellular activation mechanism for the 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.

4. The 72 kDa type IV collagenase is secreted as a complex with TIMP-2 in the presence of excess free TIMP-2.

5. Analysis of frozen human tumor samples by microdissection and zymography demonstrates that active forms of gelatinase A (MMP-2) are found at the invasive front in colorectal and breast cancer samples.

Publications:

Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Ann Rev Cell Biol 1993;9:541-73.

Kleiner DE Jr, Stetler-Stevenson WG. Structural biochemistry and activation of matrix metalloproteases. Curr Opin Cell Biol 1993;5:891-7.

Mohtai M, Smith RL, Schurman DJ, Tsuji Y, Torti FM, Hutchinson NI, Stetler-Stevenson WG, Goldberg GI. Expression of 92-kD type IV collagenase/gelatinase (gelatinase B) in osteoarthritic cartilage and its induction in normal human articular cartilage by interleukin 1. J Clin Invest 1993;92:179-85.

Stetler-Stevenson WG, Liotta LA, Kleiner DE Jr. Extracellular matrix in tumor invasion and metastasis. FASEB J 1993;7:1434-41.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09179-06 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Extracellular Matrix Pathology Section	LP NCI
OTHER:	J. Ray	Staff Fellow	LP NCI
	J. McClanahan	Medical Technologist	LP NCI
	E. Toschi	Fogarty Fellow	LP NCI
	M. Corcoran	IRTA Fellow	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Extracellular Matrix Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. Our studies have shown that TIMP-2 transcription is regulated independently of both TIMP-1 and TIMP-3 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 identified an RFLP in the TIMP-2 gene and are investigating LOH in human colorectal cancer.

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.

Current studies are aimed at investigating the mechanism of progelatinase A/TIMP-2 complex formation. In addition, cellular localization of TIMP-2 expression is being studied utilizing immunofluorescence microscopy.

Major Findings:

1. Studies of the transcription of TIMP-2 mRNA reveal that TIMP-2 is regulated independently from TIMP-1 and TIMP-3.

2. TIMP-2 inhibits tumor cell invasion through a reconstituted basement membrane *in vitro*.

3. TIMP-2 inhibits endothelial cell proliferation in response to bFGF mitogenic stimulation

4. TIMP-2 inhibits endothelial tube formation as reconstituted basement membranes.

5. TIMP-2 has erythroid-potentiating activity.

Publications:

Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Ann Rev Cell Biol 1993;9:541-73.

Murphy AN, Unsworth EJ, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. J Cell Physiol 1993;157:351-8.

Stetler-Stevenson WG, Liotta LA, Kleiner DE Jr. Extracellular matrix in tumor invasion and metastasis. FASEB J 1993;7:1434-41.

Woodhouse E, Hersperger E, Stetler-Stevenson WG, Liotta LA, Shearn A. Increased type IV collagenase in lgl-induced invasive tumors of Drosophila. Cell Growth Differ 1994;5:151-9.

Ray JM, Stetler-Stevenson WG. TIMP-2 expression modulates human melanoma cell adhesion and motility. Ann NY Acad Sci (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09387-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Kinetic Characterization of the Activation of Gelatinase A

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

PI:	D. Kleiner	Expert; Acting Chief, Postmortem Pathology Section	LP NCI
OTHER:			
	W. Stetler-Stevenson	Chief, Extracellular Matrix Pathology Section	LP NCI
	M. Buck	Clinical Associate	LP NCI
	O. Malykh	Special Fellow	LP NCI

COOPERATING UNITS *(if any)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are investigating the in vitro mechanism of activation of the matrix metalloproteinase gelatinase A. Activation of this enzyme is a critical control step in determining the amount of matrix degradation in vivo. By understanding, in detail, the in vitro mechanism of activation, we hope to develop targets for therapeutic intervention. From previous studies, we know that this enzyme is secreted as a latent pro-enzyme, often in complex with its specific inhibitor, tissue inhibitor of metalloproteinases-2, TIMP-2. Latency of the pro-enzyme is maintained by preservation of a covalent bond between a cysteine sulfhydral and the active site zinc atom. Physical disruption (denaturation) or chemical cleavage (organomercurial) of this bond result in a series of events which end in loss of 80 amino acids from the amino-terminus and development of proteolytic activity. In vivo, activation appears to occur by a cell-surface associated mechanism that gives the same result as in vitro activation. Therefore, a clear understanding of the order and rate of events in vitro will also allow better understanding of the in vivo mechanism.

In order to study the kinetics of activation, it was necessary to develop a method of quantifying different enzyme forms during the course of an activation reaction. We adapted the method of zymography (a PAGE system in which the substrate gelatin is incorporated into the gel) to be used in a quantitative fashion.

Major Findings:

1. Under conditions of neutral pH and at 37°C, progelatinase A and progelatinase A/TIMP-2 complex are stable and do not autoactivate.

2. After organomercurial activation, gelatinase A (in the absence of TIMP-2), rapidly autodegrades to small inactive fragments.

3. The activated gelatinase A/TIMP-2 complex is stable to autodegradation.

4. Quantitative zymography may be used to detect picogram quantities of the three enzyme forms observed during the course of activation: the 72, 64 and 62 kDa forms.

5. Activation of progelatinase A with APMA results in enzymatic self-cleavage that is independent of enzyme concentration. This implies an entirely intramolecular self-cleavage.

6. Addition of TIMP-2 results in inhibition of the rate of self cleavage in a manner analogous to its inhibition of other enzymatic substrate cleavage reactions.

7. Furthermore, TIMP-2 changes the mechanism of the self-cleavage so that cleavage is dependent on the enzyme concentration, implying an intermolecular step during the reaction.

Publications:

Kleiner DE, Tuuttila A, Tryggvason K, Stetler-Stevenson WG. Stability analysis of latent and active 72 kDa type IV collagenase: the role of tissue inhibitor of metalloproteinases-2 (TIMP-2). Biochemistry 1993;32:1583-92.

Kleiner DE, Stetler-Stevenson WG. Structural biochemistry and activation of matrix metalloproteinases. Current Opin Cell Biol 1993;5:891-7.

Stetler-Stevenson WG, Liotta LA, Kleiner DE. Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J 1993;7:1434-41.

Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: Detection of picogram quantities of gelatinases. Anal Biochem 1994;218:325-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09388-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Genetic Alteration of TIMP-2 Modulates the Invasive Phenotype

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

PI:	J. Ray	Senior Staff Fellow	LP NCI
OTHER:	W. Stetler-Stevenson	Chief, Extracellular Matrix Pathology Section	LP NCI
	J. McClanahan	Medical Technologist	LP NCI
	R. Hewitt	Fogarty Fellow	LP NCI

COOPERATING UNITS *(if any)*

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Interaction of cells with the extracellular matrix (ECM) plays an important role in the regulation of cell behavior. Formation of adhesive contacts leads to transduction of signals into the cell and results in altered gene expression and modulation of the cellular phenotype. Specific adhesive interactions of cells with the fibronectin and vitronectin receptors modulate expression of ECM-degrading metalloproteases. These proteases are involved in the acquisition of the invasive phenotype by a number of cell types. The activity of matrix metalloproteases (MMPs) are down-regulated by endogenous inhibitors referred to as tissue inhibitors of metalloproteases (TIMPs). Alterations in the balance between MMPs and TIMPs have been shown to alter cellular invasion through effects on matrix degradation.

Our results demonstrate that exogenous rTIMP-2 and anti-gelatinase A antibodies can be used to inhibit endogenous gelatinase A activity of A2058 human melanoma cells, and that inhibition of this protease results in enhanced cellular adhesion, and decreased motility on fibronectin and gelatin. To further explore this phenomenon, we have used a retroviral infection vector to over- or underproduce TIMP-2 in human melanoma A2058 cells. Our results indicate that altering the production of TIMP-2 modulates not only proteolysis of the extracellular matrix, but also the adhesive and spreading properties of the cells, the motility of cells on and through matrix components, and results in changes in cell morphology. In addition, cells over- or underproducing TIMP-2 exhibit decreased tumorigenic potential in nude mice. These effects of TIMP-2 appear to be mediated by inhibition of gelatinase A activity. We conclude that gelatinase A, in addition to contributing to proteolysis of ECM components, also functions to proteolyze cell surface components that are necessary for attachment of A2058 cells to the ECM. These results implicate the MMPs and TIMPs in all aspects of the cellular invasion cascade. This data support the hypothesis that highly invasive cell lines establish a balance of MMPs and inhibitors that is optimal for invasion, and alteration of this balance in either direction results in perturbation of the invasive phenotype.

Major Findings:

1. Inhibition of gelatinase A activity results in increased adhesion and decreased motility of A2058 cells.

2. A2058 cells that overproduce TIMP-2 exhibit increased adhesion to a variety of matrix components, while cells that underproduce TIMP-2 exhibit decreased adhesion.

3. Either over- or underexpression of TIMP-2 results in decreased chemotaxis of A2058 cells on gelatin, type IV collagen and fibronectin, and decreased invasion through Matrigel.

4. Alteration in the balance of gelatinase A and TIMP-2 in A2058 cells results in decreased growth of subcutaneous tumors in nude mice.

5. Alterations in TIMP-2 production appear to correlate with changes in the morphology of the infectant cell lines. Clones expressing elevated levels of TIMP-2 are significantly more spread and have more numerous sites of peripheral cell attachment than A2058 controls. In contrast, clones expressing antisense TIMP-2 mRNA and decreased TIMP-2 protein are smaller, elongated, spindled in appearance and have fewer attachment sites.

Publications:

Ray JM, Stetler-Stevenson WG. TIMP-2 expression modulates human melanoma cell adhesion and motility. Ann NY Acad Sci (in press)

Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Ann Rev Cell Biol 1993;9:541-73.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09390-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Effect of Altered Production of TIMP-2 on Gene Expression in Metastasis

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

PI:	J. Ray	Senior Staff Fellow	LP NCI
OTHER:	R. Hewitt	Fogarty Fellow	LP NCI
	N. Novas	Summer IRTA	LP NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.7	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have used a retroviral vector to over- and under- express TIMP-2 in human melanoma A2058 cells and have demonstrated that altering the production of TIMP-2 modulates not only proteolysis of the extracellular matrix, but also the adhesive and spreading properties of the cells, the motility of cells on and through matrix components, and results in changes in cell morphology. These cells also exhibit decreased tumorigenic potential in nude mice. These results implicate the involvement of TIMPs in multiple aspects of the cellular invasion cascade. To further explore the molecular basis of altered expression of TIMP-2 that results in the morphologic and phenotypic changes, we have begun to screen the TIMP-2 recombinant cell lines for changes in gene expression relative to each other. FACS cell surface staining and Northern analysis reveals that alteration in the expression of TIMP-2 modulates expression of the vitronectin receptor, $\alpha v\beta 3$, but not the fibronectin receptor, $\alpha 5\beta 1$. In addition, we are currently utilizing the method of differential display to assess changes in gene expression at the transcriptional level in the parental and TIMP-2 over-producing cell lines. Subtraction hybridization library screening techniques will also be employed to search for differentially expressed genes. These methods will enable us to perform unbiased and extensive searches for genes that are modulated by changes in TIMP-2 expression. Significance of candidate genes will be verified by Northern analysis against reference blots of normal versus tumor tissue mRNA and primary versus metastatic tumor. We hope to isolate and characterize components involved in the development of the invasive and metastatic phenotype.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09131-10 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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OTHER:	G. Senterre	Visiting Fellow	LP NCI
	L. Wrathall	Biologist	LP NCI
	T. Simmons	Biologist	LP NCI
	N. Montuori	Special Volunteer	LP NCI

COOPERATING UNITS *(if any)*

V. Romanov, Structural Biology Section, NCI, FCRDC

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.8	1.2	1.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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 nonintegrin laminin binding proteins that are present in both normal and neoplastic tissues. All three proteins bind to the poly-N-acetyllactosamine carbohydrate structures on laminin. The 67 kDa high affinity laminin receptor (67LR) 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. It is not clear how the precursor is converted into the 67LR. The precursor contains a laminin binding site as well as a binding site for the Sindbis virus receptor. The two other nonintegrin laminin binding proteins have recently been renamed galectin-1 and galectin-3. They have molecular masses of 14 kDa and 31 kDa, respectively. Expression of galectin-3 varies in different tissues. In colorectal and breast carcinomas, galectin-3 is down-regulated, and its expression is inverse to that of the 67LR. However, in thyroid tissues, galectin-3 is not expressed in normal or benign conditions, but is present in several thyroid neoplasms. Future studies will determine if the selective use of different laminin binding proteins by cancer cells may play a functional role in the disease process.

Major Findings:

Biochemistry of the 67LR. Previous experiments suggested that the 67LR is synthesized from a cytoplasmic precursor with an apparent moleuclar 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.

Redistribution of laminin receptor antigen in response to laminin stimulation. To better understand the cellular localization of the 67LR and the 37LRP, transmission electron microscopic studies of human melanoma cells were carried out using immunogold labeling and a variety of anitbodies, including affinity purified antibodies directed agaisnt 37LRP cDNA-derived synthetic peptides, anti-67LR monoclonal antibodies that were raised against human small cell lung carcinoma cells, and monoclonal antibodies against integrin subunits. Double labeling immunocytochemistry revealed that anti-67LR monoclonal antibodies as well as anti-37LRP antibodies recognized antigens that were localized in the cytoplasm in electron dense structures. Weak cell membrane labeling was also observed. Unexpectedly, α6 and ß1 integrin subunits colocalized with the 67LR in cytoplasmic structures. After addition of soluble laminin to melanoma cells in suspension, the number of labeled cytoplasmic structures increased especially in the vicinity of the plasma membrane, and more antigen appeared on the cell surface. The data suggest that antigens recognized by anti-67LR and anti-37LRP antibodies colocalize in cytoplasmic structure with α6ß1 integrin, forming a supply of laminin binding proteins that are exported to the surface upon exposure of the cells to laminin, with a consequent increase in binding sites for the ligand.

Expression of galectin-3 in thyroid tissues. The expression of galectin-3, a 31 kDa laminin binding protein, was studied by immunohistochemistry in a spectrum of benign and malignant thyroid neoplasias and in some non-neoplastic conditions. Follicular adenomas, hyperplastic nodules, goiter, and normal thyroid tissue were negative. Neoplastic cells of papillary carcinoma were strongly positive. Other thyroid neoplasias, including anaplatic carcinomas, poorly differentiated carcinomas, medullary carcinomas, and follicular carcinomas were also positive, but with less intensity. In contrast to breast and colon carcinomas, where down-regulation of galectin-3 is a marker for carcinoma, expression of galectin-3 in thyroid tissues is a phenotypic feature of malignant thyroid neoplasms, particularly papillary carcinomas.

Publications:

Sobel ME. Differential expression of the 67 kDa laminin receptor in cancer. Sem Cancer Biol 1994;4:311-317.

Romanov V, Sobel ME, da Silva P, Menard S, Castronovo V. Cell localization and redistribution of the 67 kD laminin receptor and α6 and ß1 integrin subunits in response to laminin stimulation: an immunogold microscopy study. Cell Adhesion and Commun 1994; (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09353-04 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	G. Senterre	Visiting Fellow	LP NCI
	E. Campo	Guest Researcher	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:	PROFESSIONAL:	OTHER:
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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 galectin-3 (a 31 kDa laminin binding protein) and at genes involved in cell development and differentiation such as homoeobox (HOX) genes. To accomplish this, we have recently cloned galectin-3 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. In ovarian cancers, 67LR expression was directly correlated with poor prognosis, while expression of galectin-3 did not significantly differ amongst the different patient groups. In MCF7 cells, derived from a human breast carcinoma, we identified mRNA transcripts from several HOX genes, including A1, A4, A10, B5, B7, and C6. Several polymorphisms or mutations have been identified in the MCF7 HOX mRNA transcripts. In addition to the normal HOXB7 mRNA transcript, MCF7 breast carcinoma cells express another transcript which would translate into a shorter HOXB7 protein. The role of such alterations are being examined in breast cancer tissues.

Major Findings:

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, galectin-3, 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.

Expression of homeobox genes in human breast cancer cells. Homeobox genes, encoding transciptional regulators, act in complex regulatory cascades to control the coordinated expression of genes involved in specific developmental processes. We have asked the question whether specifc 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: HOXA1, 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 HOXC6 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. Most of the MCF7 HOXB7 cDNA clones contained a transversion from G to T within the penultimate codon of the presumed coding region, resulting in a translational termination signal TAA, and a derived polypeptide that is truncated by two glutamic acids at the carboxyterminus. This polymorphism appears to be specific to MCF7 cells. The expression of HOXB7, as well as of the other identified HOX mRNAs, varies in different human breast-derived cell lines. The physiologic significance of these variations and polymorphisms/mutations are under investigation.

Publications:

van den Brule F, Berchuck A, Bast RC, Liu F-T, Gillet C, Sobel ME, Castronovo V. Differential expression of the 67 kD laminin receptor and 31 kD human laminin binding protein in human ovarian carcinomas. Eur. J. Cancer 1994; (in press)

van den Brule F, Price J, Sobel ME, Lambotte R, Castronovo V. Inverse expression of two laminin binding proteins, 67LR and galectin-3, correlates with the invasive phenotype of trophoblastic tissue. Biochem Biophys Res Commun 1994; (in press)

Castronovo V, Kusaka M, Chariot A, Gielen J, Sobel M. Homeobox genes: potential candidates for the transcriptional control of the transformed and invasive phenotype. Biochem Pharm 1994;47:137-43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09368-03 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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 Sec.	LP NCI
OTHER:	L. Wrathall	Biologist	LP NCI
	T. Simmons	Biologist	LP NCI
	A. Alag	Special Volunteer	LP NCI
	N. Montuori	Special Volunteer	LP NCI
	E. Campo	Guest Researcher	LP NCI
	E. Muro	Visiting Fellow, Surg. Path.	LP NCI
	J. Sanz-Ortega	Guest Researcher, Surg. Path.	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:	PROFESSIONAL:	OTHER:
1.0	0.3	0.7

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

D

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

A molecular pathology resource center has been 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 provides a resource with tissue specimens for the analysis of gene expression in neoplasia. In addition, bench experience and didactic training in molecular techniques are provided, including nucleic acid and protein extraction, tissue handling, Northern blot, Southern blot, immunoblot, *in situ* hybridization, probe labeling, polymerase chain reaction, and ligase chain reaction. During this year, we are providing training to two graduate students and three postdoctoral fellows.

Major Accomplishments:

Users of the molecular pathology resource center are using a variety of apporaches to apply molecular techniques to the study of human cancers. Projects include the use of recombinant proteins for the study of protein structure and antibody production, the molecular cloning of calmodulin genes and the study of mRNA stability, and the detection of gene alterations in breast cancers using PCR.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09382-02 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Signal Transduction and AP-1 Transcription Factors in Mouse JB6 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; Dr. D. Ferris, Biological Carcinogenesis and Development Program, NCI, FCRDC

LAB/BRANCH

Laboratory of Pathology

SECTION

Molecular Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The AP-1 transcription factor modulates expression of genes involved in growth regulation, differentiation and neoplastic transformation. AP-1 transcription factors are protein dimers consisting of members of the *jun* and *fos* proto-oncogene families. Protein kinases in the signal transduction pathway are believed to regulate AP-1 activity. Although over a dozen kinases from several protein kinase families, including MAP kinases, have been identified which phosphorylate AP-1 proteins *in vitro*, *in vivo* associations between the kinases and AP-1 have not been proven. The purpose of this project is to seek evidence for *in vivo* interaction between AP-1 and MAP kinase proteins and to determine how such an interaction could affect gene regulation in cancer cells. Using cultured mouse epidermal JB6 cells and immunoprecipitation experiments with anti-MAP kinase and anti-AP-1 antibodies, we have detected for the first time an *in vivo* association of seven MAP kinase-related proteins with a variety of AP-1 dimers. ERK-2 and several apparently novel MAP kinase-related proteins are among the species which interact with AP-1. The large number of MAP kinase-related proteins associated with AP-1 implicate them along an important and heavily utilized pathway for regulating signal transduction to control gene expression. Combinatorial association between MAP kinase related proteins and AP-1 dimers could potentially create numerous distinct complexes that could regulate diverse genes.

Major findings:

1. Seven MAP kinase-related proteins (MKRPs) in JB6 cells interact *in vivo* with AP-1, as determined by peptide mapping and two dimensional electrophoretic analyses of proteins co-precipitated with AP-1 antigens. These MKRPs include species designated as p96, p65, p49, p40, p38, p36, and p33.

2. The MKRP p40 which interacts *in vivo* with AP-1 is the MAP kinase protein ERK-2. ERK-2 has been cloned and sequenced and its structure has been determined crystallographically by other laboratories.

3. A variety of AP-1 dimers associate with MKRPs, including Jun-Fos, Jun-Fra-1 and Jun-Fra-2 complexes.

4. MKRPs associated with AP-1 have specific *in vitro* kinase activity.

5. Several MKRPs display elevated levels in mouse JB6 cells susceptible to promotion of neoplastic transformation by tumor promoters compared to cells resistant to neoplastic transformation by tumor promoters.

Publications:

Bernstein LR, Ferris DK, Colburn, NH, Sobel ME. A family of mitogen-activated protein kinase-related proteins interacts *in vivo* with activator protein-1 transcription factor. J Biol Chem 1994;269:9401-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09386-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Expression of Calmodulin in Human Cancers

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 Fellow	LP NCI
	A. Alag	Special Volunteer	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:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Calmodulin is a mediator of cellular responses to calcium fluxes. This high affinity intracellular calcium binding protein activates target enzymes that affect many different cellular processes. In mammalian species, three genes (calmodulin-I, II, III) encode an identical calmodulin protein, however the mRNA transcripts for the three different genes have distinct 5' and 3' untranslated regions, suggesting that they may be regulated differently post-transcriptionally. By Northern blot analysis, calmodulin-I has two mRNA transcripts of approximately 1.6 and 4.4 kb. The purpose of this project was to determine the molecular mechanism for the two calmodulin-I mRNA transcripts in human cells, and to test the hypothesis that calmodulin-I mRNAs are differentially expressed in cancer cells. We have cloned and completely characterized calmodulin-I cDNA clones that encompass 4200 base pairs of sequence. We have determined that the alternate use of polyadenylation signals in the 3' untranslated region of the mRNA is the mechanism for the two calmodulin-I transcripts in human cells. Expression of both calmodulin-I mRNAs is decreased in human colorectal carcinomas compared to adjacent normal mucosa, suggesting that colon cancer cells may respond to calcium in a different manner than normal colonic mucosa.

Major Findings:

Cloning of full length calmodulin-I cDNA. Previously, 850 bases of calmodulin-I cDNA had been cloned, including the complete coding region and partial 5' and 3' untranslated regions. A human melanoma cDNA library was screened with a calmodulin-I coding region cDNA probe and several clones were characterized and completely seqeunced. We have obtained 4200 bases of sequence from overlapping clones, and have proven that they are contiguous sequences by reverse transcriptase-polymerase chain reaction assays. Two polyadenylation signals, approximately 2600 bases apart from each other, were identified. Northern blot hybridization experiments, using probes from 5' and 3' regions of the cDNA clones, proved that the two calmodulin-I mRNA transcripts of 1.6 and 4.4 kb are the result of alternate use of polyadenylation signals.

Calmodulin-I mRNAs are decreased in colorectal cancers. Northern blot anaylsis of matched human colorectal carcinomas with normal adjacent mucosa demonstrated that calmodulin-I mRNAs are less expressed in the cancers than in the normal tissues. This suggests that colorectal cancer cells may respond to calcium in a different manner than normal colonic cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09163-07 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Signal Transduct. & Prevent. Unit	LP NCI
OTHER:	W. Jacobs	General Fellow	LP NCI
	K. Cole	General Fellow	LP NCI
	J. Spoonster	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, TIM Section	LP NCI

COOPERATING UNITS *(if any)*

Kevin Gardner, LP, NCI; FCRDC animal contract unit; C. Felder, LCB, NIMH

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.8	2.7	2.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews D

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

CAI (carboxyamido-triazole) has served as a unique tool to study novel cell signaling pathways and their biologic consequences. With the successful completion of the structure function analysis of CAI and allowance of U.S. patent claims on CAI matter, the focus of the laboratory has moved to the molecular and protein targets of CAI interaction. The molecular dissection of CAI demonstrated that CAI inhibition of calcium-mediated signaling events is concordant with inhibition of malignant proliferation in vitro and that the complete molecule is necessary for activity. CAI-resistant A2058 cells which were developed over the last 4 years have been used for a molecular subtraction analysis. This project has yielded at least 3 genes which are upregulated in the resistant cells. Partial sequence and GeneBank analysis has determined that 2 are novel genes. Work on the complete gene sequence and activity is ongoing and antipeptide antibodies to one of the gene products are being developed. CAI is also being used as a tool to characterize calcium-mediated regulation of gene expression. We have shown that MMP-2 regulation is CAI- and SKF96365-sensitive and that reduction in MMP-2 occurs at the level of gene expression. New findings suggest a role for CAI in regulation of transcription factor interactions.

We are approaching identification of the protein targets of CAI through several means. Early promising results have been observed using a column chromatography shift assay. Affinity dye columns are under investigation, and new detection methods are being developed. These include photoaffinity crosslinking using the intrinsic ability of CAI to absorb at 263 nm, and labelling of CAI by placement of biotin or FITC on a carbon chain linker to minimize alteration of the tertiary structure and thus activity of the molecule.

Further signal transduction studies have confirmed the calcium-mediated mechanism of action of CAI and have identified a role for calcium influx regulation of tyrosine phosphorylation. The inhibition of bFGF-mediated tyrosine phosphorylation in endothelial cells by CAI is a potential mechanism underlying our findings of profound inhibition of angiogenesis by CAI. It may also drive the inhibition of myelopoiesis and erythropoiesis as shown in our studies of the bone marrow effects of CAI.

Major Findings:

1. 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 in concentrations ranging from 10-40 μM; a > 75% growth inhibition of wild type A2058 cells is observed with exposure to CAI 10 μM. Both molecular and cellular analyses of the resistant cells are ongoing. Subtraction cDNA analysis of A2058 cells resistant to 20 μM CAI (A2058-20R) against wild type A2058 cells (WT) has identified demonstrated several novel gene products. Library screen yielded 35 non-overlapping clones of which 20 have been analyzed by Northern blot and 3 encode genes which are increased in 20R cells. CAIR-1 (21DBB) encodes a 2.8 kb message of which 1400 3' basepairs have been sequenced. This gene is novel by GeneBank comparison and has several amino acid sequences which have striking homology to the newly characterized SH3 binding domains. This gene is normally highly expressed in heart and skeletal muscle, organs which have significant calcium functional requirements. A patent has been filed on this gene product and further investigation of the stucture and function of this novel cDNA is ongoing. One of the other genes also is unique and the third is now being sequenced. Biologic analysis of the resistant lines will include evaluation of doubling time, MMP-2 production, migration, signal transduction parameters, and clonigenicity in soft agar and tumorigenicity in nude mice.

2. 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 significantly inhibits human umbilical vein endothelial cell adhesion, motility, proliferation, and MMP-2 production in the same effective concentration range as tumor cells. The effect on MMP-2 is at the level of expression as we have shown for tumor cell MMP-2. Striking inhibition of neovascularization was seen in chick chorioallantoic membrane and associated with die back of primary venules. An inactive CAI analog was found to have no inhibitory effect on angiogenesis. Investigations have demonstrated that CAI treatment inhibits basic fibroblast growth factor stimulated tyrosine phosphorylation to basal levels. Studies are ongoing to identify the CAI-sensitive phosphorylation substrates.

3. Effect of calcium-mediated signaling on gene transcription. We have studied the effect of calcium homoestasis on several different gene systems. First, CAI treatment inhibited MMP-2 activity by zymogram analysis and calcium-influx sensitive MMP-2 production. Northern analysis demonstrated decreased gene expression after CAI exposure. Next, we investigated its role in AP-1 sensitive systems and demonstrated CAI inhibition of AP-1 mediated activity such as in interleukin-2 expression. Further lymphocyte signaling studies are ongoing with Dr. Kevin Gardner. Lastly, a paradoxical effect of time dependent stimulation or inhibition of production of the VL-30 enhancer has been shown in collaboration with Karin Rodland, PhD (Oregon Health Science University). This enhancer is regulated by intracellular calcium concentrations and growth factor stimulation of calcium mobilization.

4. Effects of signal transduction therapy on hematopoiesis. Collaboration with Donna Volpe, PhD (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 IC50 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 almost 10-fold higher IC50 values. The formation of bone marrow stimulating-cytokines after pokeweed mitogen exposure was also affected by CAI exposure. Studies are ongoing with the analogs to determine if differential effects are observed. A manuscript has been completed and submitted.

5. Identification and cloning of CAI binding site. Due to the unreactive nature of CAI, attempts to modify the structure with detectable probes has been limited. Biotinylated CAI made on contract did not perform in either binding or far-Western studies. Current strategies are focused towards identification of the binding protein. We are investigating several chromatographic approaches. Column shift assays in which the elution time of radiolabelled CAI is altered after incubation with cellular proteins is in progress. Dyes that may have chemical similarity to CAI are being used in affinity columns where CAI will be used to selectively elute its binding proteins. Experiments to take advantage of the strong absorption of CAI at 263 nm will determine whether CAI can be photoaffinity crosslinked after exposure to UV light without the requirement for chemical modification to an azide compound. The carbonyl of the benzophenone should be reactive, however, the chlorines may cause steric hinderance. This approach is being tested. Lastly, native far-Westerns using isoelectricfocusing will be tried. Detection will use phosphorimager analysis to overcome the solubility of CAI in scintillation fluors.

Publications:

Kohn EC. Development and prevention of metastasis. Anticancer Res 1993;13:2553-60.

Kim YS, Liotta LA, Kohn EC. Cancer invasion and metastasis. Hosp Pract May 1993;92-6.

Kohn EC, Liotta LA. Signal transduction inhibitor compounds. Patent application #07/985,402. Filed December 4, 1992. Matter claims allowed 4/94.

Cole KA, Kohn EC. Calcium-mediated signal transduction: biology, biochemistry, and therapy. Cancer Metast Rev 1994;13:33-41.

Alessandro R, Kohn E. Metastasis and cancer prevention targets. Comprehensive Textbook of Oncology Update Series. Baltimore: Williams & Wilkins, 1993;1:1-6.

Kohn EC, Felder CC, Jacobs W, Holmes KA, Day A, Freer R, Liotta LA. Structure function analysis of signal and growth inhibition by carboxyamido-triazole, CAI. Cancer Res 1994;54:935-42.

Kohn EC, Liotta LA. Method for inhibiting metalloproteinase expression. Patent application #08/123/164. Filed September 17, 1993.

Kohn EC, Alessandro R, Spoonster J, Liotta LA. Angiogenesis: role of calcium-mediated signal transduction. Proc. Natl. Acad. Sci. USA (in press)

Kohn EC, Reed E, Liotta LA. Combination therapy using signal transduction inhibitors and cytotoxic agents. Patent application filed March 14, 1994.

Kohn EC, Kim YS, Liotta LA. DNA encoding CAI resistance proteins and uses thereof. Patent application filed March 14, 1994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09374-02 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Novel Signaling Pathways

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

PI:	E. Kohn	Chief, Signal Transduction and Prevention Unit	LP NCI
OTHER:	W. Jacobs	General Fellow	LP NCI
	R. Alessandro	Visiting Fellow	LP NCI
	R. Wersto	NRC Fellow	LP NCI

COOPERATING UNITS *(if any)*

Christian Felder, Ph.D., Laboratory of Cell Biology, NIMH

LAB/BRANCH

Laboratory of Pathology

SECTION

Signal Transduction and Prevention Unit

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Novel signaling events with calcium regulation have been investigated. We have demonstrated an endogenous muscarinic receptor (mAChR) on A2058 human melanoma cells which results in calcium influx and arachidonic acid release. Binding studies indicated that the receptor is of low abundance (~40 fmol/mg membrane protein) and high affinity. Activation of the receptor also results in pertussis toxin-insensitive inhibition of cAMP production and no generation of inositol phosphates. Up modulation of arachidonic acid release in response to carbachol was observed after at least 30 min pretreatment with TNFα; this was diminished by treatment with cycloheximide suggesting the requirement for new protein synthesis. Activation of this mAChR results in inhibition of soft agar colonization of A2058 cells, suggesting an anti-oncogenesis role as we have previously shown for mAChR in CHO cells. Molecular characterization of this receptor is underway using PCR to amplify the third cytoplasmic domain of the A2058 mAChR as this region has the most diversity between subtypes.

We have also initiated investigation into signaling events associated with other ion channels, beginning with chloride homeostasis. We have observed modulation of cAMP-mediated Cl- efflux through the CFTR pathway by CAI; this channel has previously reported to be calcium insensitive. The effect requires 5-10 min pretreatment and similar findings were observed with SKF96365 or BAPTA treatment, suggesting a heretofore undescribed effect of calcium homeostasis on Cl- efflux. Further studies are underway to characterize whether this is indeed a calcium-related effect or a novel mechanism of action for CAI.

Major Findings:

1. Identification of calcium-regulated signaling pathways. We have previously demonstrated efficacy of CAI in regulation of calcium-sensitive release of arachidonic acid, calcium influx, and tyrosine phosphorylation in several systems. These studies have resulted in the description of a novel muscarinic receptor (see below) and have now reached into other ion channels. The CFTR Cl- efflux channel has been reported to be calcium-independent in its regulation and that there is an independent calcium-dependent Cl- efflux route in some cells. We have observed down-modulation of CFTR-mediated Cl- efflux after addition of CAI with activation of a Cl-efflux pathway which is independent of CFTR in cells with and without known calcium-driven Cl-efflux pathways. Similar effects are seen when SKF96365, BAPTA, or calcium-free conditions are used. This effect is reversible and both time and dose dependent. Further studies are ongoing to investigate this novel channel effect.

2. Biochemical characterization of endogenous muscarinic receptors (mAChR) on the A2058 human melanoma cell. A functional mAChR has been demonstrated by its ability to stimulate calcium influx and internal release. Further studies have shown that this receptor stimulates 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 observed. Activation of this receptor inhibited forskolin-stimulation of cAMP production, a function of even-numbered muscarinic receptors. The inhibition of forskolin-stimulated cAMP by receptor activation could not be abrogated by cell treatment with pertussis toxin as is seen for the even-numbered receptors. Recent studies have indicated that cellular pretreatment with TNFα results in an augmentation of the release of arachidonic acid in response to agonist; A2058 cells do not express both TNFα receptors on their surface. These collective 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. Binding experiments indicated extremely low abundance of muscarinic receptor in the A2058 cells, 40 fmol/mg A2058 membrane protein, making pharmacologic characterization of the receptor(s) difficult. A biologic effect in response to activation of this receptor has been observed in the form of inhibition of colonization potential in soft agar experiments suggesting that this receptor may have an anti-oncogenic potential as we have previously shown for mAChR in CHO cells.

3. Molecular identification of the novel mAChR. 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 selected muscarinic receptor. The third cytoplasmic domain of the receptor is targeted as this is the least conserved region of the muscarinic receptor family. PCR amplification using oligonucleotides containing coding sequence from the highly conserved m5 muscarinic receptor transmembrane domains of A2058 mRNA and control RNA from the m5 receptor yielded fragments. Further amplification is ongoing to isolate a larger fragment of the third cytoplasmic domain for sequence analysis and subsequent cloning strategies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09375-02 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Signal Transduction and Prevention Unit	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, Clinical Pharmacology Branch, DCT, NCI: Developmental Therapeutics Program, DCT, NCI; Cancer Therapy Evaluation Program, DCT, NCI; Division of Cancer Prevention and Control

LAB/BRANCH

Laboratory of Pathology

SECTION

Signal Transduction and Prevention Unit

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	0.3	0.7

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 Phase I clinical trial (MB281) of orally administered CAI for patients with refractory solid tumors began accrual in March 1992 and is nearing completion. Eligible patients have received CAI test doses in three formulations: PEG-400 solution or gelatin capsule, and micronized powder capsules. The test dose pharmacokinetic analysis is followed by daily administration of CAI with further pharmacokinetics. Since initiation, 38 patients have received CAI. Plasma levels have been measurable and have been between 0-10 μM over dose levels ranging from 100 mg/m2/d to 330 mg/m2/qod. A long half life (>24 hr) and a linear relationship between AUC and increasing dose have been demonstrated. No significant differences in pharmacokinetics have been seen between the formulations. Side effects of nausea with vomiting, easily treated with antiemetics, and fatigue and mood changes are mild and appear to be drug related. Three episodes of sensory peripheral neuropathy and one episode of neutropenia have been observed and were reversible. Disease stabilization (2-7 months) has been observed in 46% of evaluable patients with a pancreatic cancer patient progressing at 5 months and a renal cell cancer progressing at 7 months of therapy. Preclinical efficacy and toxicity studies for chemoprevention use of CAI have begun and are promising. In vitro supra-additive efficacy of the combination of CAI preceeding paclitaxel has been seen; a Phase I clinical trial will be starting to test this combination.

Major Findings:

1. Phase I clincal trial. The Phase I clinical trial of orally administered CAI has accrued 38 patients on 5 dose and schedule levels with liquid and gelatin capsule formulations. A long half life, dose-dependent AUC, and dose-independent clearance have been seen in pharmacokinetic analysis of test dose blood concentrations. CAI has been well tolerated with grade I-II nausea and vomiting, fatigue and mood disorders (CTEP criteria). Dose-limiting toxicity was grade III sensory neuropathy and grade IV neutropenia at 330 mg/m2/qod (liquid) and gastrointestinal intolerance, grade II but compliance-limiting at 125 mg/m2/d (gelcap). Dose escalation is continuing with liquid at 150 mg/m2/d and upon completion of the current study, will begin anew with the new micronized powder capsule formulation. Disease stabilization has been observed in 16/35 evaluable patients with durations of 2-7 months in patients with renal cell carcinoma, pancreatic cancer, colon cancer, and non-small-cell lung cancer. Phase II studies are planned and CAI will be proposed for use in the adjuvant setting in an upcoming Phase II clinical trial of newly diagnosed ovarian cancer patients.

2. Metabolite analysis. We have observed unexpected peaks on HPLC chromatography of blood and urine of patients on CAI. Characterization of a urinary peak is underway purified from urine of several patients. GC mass spectroscopy and NMR studies are being done in collaboration with Dr. Yeh of NIDDK. Studies in collaboration with Dr. Jerry Collins of the FDA have demonstrated hepatic metabolism of CAI through the P450 system consisting of hydrolysis followed by glucuronidation. A manuscript of those findings has been completed for submission.

3. Chemoprevention development. CAI was presented to the Chemoprevention Working Group/Chemoprevention Decision Network and has been accepted for preclinical investigation. Preliminary studies have found efficacy of CAI at low concentrations in intermediate marker studies including rat tracheal epithelial cell transformation assays and soft agar cloning assays. In the colon cancer chemoprevention model, oral administration of CAI to azoxymethane-treated rats markedly reduced the incidence of aberrant colon crypts but did not affect the promotion phase. Oral efficacy studies are ongoing for breast cancer prevention models (MNU) and preliminary results are encouraging. Further studies for colon cancer prevention are planned and preclinical toxicity studies are to start. Discussion is ongoing for clinical protocol development and IND cross-filing.

4. CAI and paclitaxel combination. In vitro studies of the combination of CAI and paclitaxel (Taxol®) in human ovarian cancer and breast cancer cell lines has shown selective efficacy in a schedule-dependent fashion. The selectivity of this response is suggested. An antagonistic interaction between CAI and carboplatin was found, whereas an at least additive effect was seen with CAI and paclitaxel. A 24 hr exposure of A2780 human ovarian cancer cells or their cisplatin-resistant subline A2780-CP70 to CAI at the IC25 level followed by a 24 hr exposure to a range of concentrations of paclitaxel yielded supra-additive inhibition of colony formation. Only an additive effect was seen when

paclitaxel was given prior to CAI. An additive effect of CAI followed by paclitaxel was observed for the MDA-435 human breast cancer cell line. A Phase I clinical protocol has been presented to the IRB and CTEP and awaits an ongoing nude mouse toxicity study. This protocol will administer CAI at 100 mg/m2 for 8 days followed by a 3 hr infusion of paclitaxel in increasing doses. It will be open to all solid tumor histologies. Further laboratory studies are ongoing to identify and characterize the mechanism of the synergy between CAI and paclitaxel.

Publications:

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Jerian SM, Sarosy GA, Link CJ, Fingert HJ, Reed E, Kohn EC. Incapacitating autonomic neuropathy precipitated by taxol. Gynecol Onc 1993;51:277-80.

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Reed E, Janik J, Bookman MA, Rothenberg M, Smith J, Young RC, Ozols RF, VanderMolen K, Kohn E, Jacob JL, Cornelison TL. High dose carboplatin and rhGM-CSF in advanced stage recurrent ovarian cancer. J Clin Oncol 1993;11:2118-26.

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Spoonster JR, Kohn EC. CAI and signal transduction: a novel approach to cancer treatment and prevention. Contemporary Oncol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00550-14 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	D. Longo	Senior Investigator	BRMP NCI
	M. Raffeld	Senior Staff Fellow	LP NCI
	M. Stetler-Stevenson	Senior Staff Fellow	LP NCI
	D. Kingma	Senior Clinical Investigator	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:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

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:

We have continued our studies correlating histopathologic findings with immunophenotype and molecular characterization in malignant lymphoma. We have pursued our analyses of non-Hodgkin's lymphomas associated with Hodgkin's disease including composite and sequential Hodgkin's disease/non-Hodgkin's lymphoma. It had been suggested that non-Hodgkin's lymphomas occurring after Hodgkin's disease were most likely secondary to the underlying immunodeficiency of Hodgkin's disease. Therefore, it had been postulated that such tumors might harbor Epstein-Barr viral (EBV) sequences. Using an *in situ* hybridization technique and the Eber-1 RNA probe, we studied 12 cases of composite NHL and HD, two patients with NHL who simultaneously also had HD involving a different site, and 14 NHLs arising in patients who previously had HD, and 7 NHLs from patients who subsequently developed HD. EBV sequences were identified most frequently in composite NHL and HD (42%). Moreover, in 4 of the 5 positive cases both the NHL and HD components were concordant for EBV expression, suggesting that they may have arisen from the same EBV infected progenitor cell. We identified EBV in only 2 of 14 NHLs following HD. These results suggest that EBV plays a minimal role in the NHLs associated with HD, with the exception of composite lymphomas. While Hodgkin's disease associated immune defects may be involved in the pathogenesis of NHLs following HD, EBV was not implicated as a causative agent.

Studies of MALT lymphoma were pursued, and support the concept that MALT lymphomas represent a distinct clinicopathologic entity. A MALT lymphoma of the kidney was described. Additionally, recurrent cytogenetic abnormalities have been identified in 2 cases of MALT lymphoma.

A broadly based international study to arrive at a consensus classification for the malignant lymphomas was completed. This consensus approach embraces immunologic and molecular concepts, and delineates distinct clinicopathologic entities of T- and B-cell origin. It was proposed that at an international conference that this classification scheme be adopted for clinical trials.

Publications:

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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 1993;117:392-9.

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Raffeld M, Jaffe ES. Avidin-biotin labelling of cellular antigens in cryostat-sectioned tissue. In: Javois LC, ed. Immunocytochemistry methods and protocols. (in press)

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Mason DY, Banks PM, Chan J, Cleary ML, Delsol G, de Wolf-Peeters C, Falini B, Gatter K, Grogan TM, Harris NL, Isaacson PG, Jaffe ES, Knowles DM, Müller-Hermelink HK, Pileri S, Ralfkiaer E, Stein H, Warnke R. Nodular lymphocyte predominance Hodgkin's disease. A distinct clinicopathological entity. Am J Surg Pathol 1994;18:526-30.

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Jaffe ES. Invited commentary. Non-Hodgkin's lymphomas in the older person. A review. Abstracts of Clinical Care Guidelines 1994;6:7-8.

Whang-Peng J, Knutsen T, Jaffe E, Raffeld M, Zhao WP, Duffey P, Longo DL. Cytogenetic study of two cases with lymphoma of mucosa associated lymphoid tissue (MALToma). Cancer Genetics and Cytogenetics (in press)

Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary M, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Müller-Hermelink HK, Pileri SA, Piris MA, Ralfkiaer E, Warnke RA. A revised European-American Classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00855-12 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	D. Kingma	Sr. Clinical Investigator	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:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

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:

Studies regarding the role of Epstein-Barr virus (EBV) in the pathogenesis of angiocentric immunoproliferative lesions and angiocentric lymphoma were pursued. Although lymphomatoid granulomatosis had been proposed as a form of T-cell lymphoma, using combined immunohistochemistry and *in situ* hybridization, EBV sequences were localized to B cells, and not T cells. The EBV-affected B cells were associated with a prominent T-cell reaction, and T cells predominated in tissue sections. Nevertheless, clonality of the T-cell reaction could not be shown. Polymerase chain reaction for V-J IgH sequences demonstrated clonal immunoglobulin gene rearrangement in the majority of cases studied. These findings suggest that lymphomatoid granulomatosis is a B-cell lymphoproliferative disorder with an exuberant T-cell reaction. These findings contrast with those of angiocentric lymphoma in which EBV sequences can be localized to cells with a T/NK phenotype.

The viral pathogenesis of Kikuchi-Fujimoto disease was pursued using both *in situ* hybridization and the polymerase chain reaction. The findings suggest that EBV and human herpevirus type 6 are not implicated in the pathogenesis of Kikuchi-Fujimoto disease.

Parallel case control studies in Jamaica and Trinidad/Tobago to quantify the role of HTLV-I in the development of non-Hodgkin's lymphoma were conducted. Overall, patients with NHL were 10 times more likely than were controls to be seropositive for HTLV-I. In both countries the association between NHL and HTLV-I was greatest for T-cell lymphomas. Among T-cell lymphomas, there was no significant difference between men and women in the association between NHL and HTLV-I, but there was a significant inverse relation between age and likelihood of HTLV-I seropositivity. B-cell lymphomas were seen predominantly in older age groups and were not associated with HTLV-I seropositivity. These findings are consistent with the hypothesis that early life exposure to HTLV-I is important for risk of subsequent ATL.

Publications:

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Parkhurst JB, Kuhls TL, Elrod JP, Sexauer CL, Jaffe ES, Peiper SC. Lymphomatoid granulomatosis (angiocentric immunoproliferative lesion) in a child with familial chronic active Epstein-Barr virus infection. Int J Pediatr Hematol/Oncol (in press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09182-06 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	H. Clark	Visiting Fellow	LP NCI
	T. Otsuki	Visiting Fellow	LP NCI
	A. Wellman	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:	PROFESSIONAL:	OTHER:
1.85	1.85	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A,B

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

We 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.

Our investigations of the Myc gene in small non-cleaved cell lymphomas have led us into a more basic study of Myc structure/function relationships. We have found that Burkitt's and AIDS-associated lymphomas with 8q24 translocations have a very high frequency of clustered mutations in the transcriptional activation domain of Myc. These mutations appear to be selected during tumorigenesis, and the mutant Myc proteins have increased growth associated activities as compared to the normal Myc protein. The mechanism(s) by which these mutations alter functional activity is being explored in collaboration with Dr. Chi V. Dang of Johns Hopkins University.

We have undertaken several new projects in the past year to explore the role of several newly described genes in lymphomas. These include the LAZ3 (BCL-6) gene (see major findings), the tumor suppressor gene p16, and the anaplastic lymphoma associated gene nucleophosmin.

Major Findings:

1. Mantle cell lymphoms is associated with rearrangement of the bcl-1 locus. Using a set of four probes, 70% of cases have a rearrangement of this genetic region. The BCL-1/Prad1 gene is overexpressed in mantle cell lymphomas and in some cases of prolymphocytic leukemia, but not in other lymphoma subtypes. This overexpression is associated with the bcl-1 region rearrangement, a consequence of the 11;14 translocation.

2. LAZ3 (BCL-6) is rearranged in approximately 25% of de novo large cell lymphomas. LAZ3 rearrangements are not specific to de novo cases as rearrangement may also be seen in 15% of follicular and transformed follicular lymphomas. Expression of LAZ3 is not appreciably higher in rearranged vs nonrearranged B-cell lymphomas. Mutations do not occur in LAZ3 coding sequences.

3. The transcriptional activation domain of the Myc gene is frequently mutated in Burkitt's and AIDS-associated lymphomas. Myc proteins with mutations in the region of58Thr and 62Ser show alterations of in vitro functional activities. Specifically, they show increased activity in transformation assays and resist p107 mediated suppression of transactivation.

Publications:

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 1993;8:2741-8.

Clark HM, Yano T, Otsuki T, Jaffe ES, Shibata D, Raffeld M. Mutations in the Myc gene in AIDS-associated and other aggressive lymphomas. Cancer Res. (in press, July 1, 1994)

Raffeld M, Yano T, Huang A, Clark HM, Otsuki T, Dang CV. Clustered mutations in the transcriptional activation domain of the Myc gene in 8q24 translocated lymphomas and their functional consequences. In Mechanisms of B-cell neoplasia 1994. Curr Topics in Microbiol Immunol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09191-05 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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1.35	1.35	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A,B

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

The 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 disease, characterized primarily by a single molecular lesion (bcl-2 translocation) and because over 70% of these low grade lymphomas will evolve into a histologically distinct high grade lymphoma.

Previously, we found that the bcl-2 gene itself was unaffected by progression, implying that other genes must be contributing to the dramatic changes in cellular morphology and biologic behavior that occur in transformation. For the past few years, we have been analyzing the role of known oncogenes and tumor suppressor genes in a large series of progressed follicular lymphomas, using a combination of molecular genetic and immunohistochemical techniques.

We have completed studies on the involvement of the Myc gene and have found acquired, progression related structural changes in this gene in approximately 10% of progressed lymphomas. We have recently completed another study on the involvement of the bcl-3 (17q22) gene and have also found abnormalities of this locus in 10-15% of the progressed lymphomas. In contrast to the situation for Nyc, these changes are not temporally associated with progression and we have concluded that alterations of this gene do not directly result in progression. We also completed a study of the role of the p53 gene in progression using SSCP and sequencing. We have found that approximately 30% of transformed follicular lymphomas harbor acquired p53 mutations, not present in a significant percentage of tumor cells in the antecedent low grade lymphoma. This suggests that a substantial proportion of follicular lymphomas undergo histologic progression via a pathway that involves mutation of the p53 gene. We are extending the study to incorporate the entire NCI MB110 low grade lymphoma protocol in order to see whether p53 overexpression and mutation can be used as a temporal predictor of progression. We are also currently investigating the role of MDM-2 and p21, additional genes involved in the p53 pathway. Other genes under investigation include c-rel, Rb, PCNA and p16.

Major Findings:

1. Acquired Myc gene rearrangements occur in about 10% of lymphomas undergoing histologic and clinical progression. The molecular structure of these rearrangements are different from those which commonly occur in high grade Burkitt's lymphomas.

2. BCL-3 (chr 17q22) abnormalities are present at a prevalence of approximately 10% in both transformed lymphomas and their antecedent low grade lymphoma. The relationship of this molecular lesion to progression is not clear.

3. Acquired p53 mutation is common in progressed follicular lymphomas, and is highly associated with histologic transformation.

Publications:

Yano T, Longo D, Jaffe ES, Raffeld M. Molecular analysis of the BCL-3 glocus at chromosome 17q22 in B-cell neoplasms. Blood 1993;82:1813-9.

Sander CA, Yano T, Clark HM, Harris C, Longo D, Jaffe DL, Raffeld M. p53 mutation is associated with progression in follicular lymphomas. Blood 1993; 82:1994-2004.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09372-03 LP

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Flow Cytometric Analysis of Benign and Malignant Tumors

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

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1.25	1.25	

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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.)*

In lymph node negative breast cancer, a strong association has been shown between S-phase and prognosis. We studied the effect on S-phase fraction estimates of debris compensation using four debris subtraction algorithms and three different sets of nuclear debris boundaries in a series of 185 fresh frozen breast carcinomas. Comparison of S-phase estimates indicates good correlation between algorithms modeling debris based upon nuclear slicing and fragmentation compared to modeling of debris based upon an exponentially decreasing power function. Under certain defined conditions, initial debris boundary placement did affect S-phase estimates, especially when debris was modeled using a decreasing exponential function. S-phase determinations were also affected by the CV of the G1/0 peak and the relative amount of debris. Contrary to previous reports, our data also indicates the need for debris compensation even when the debris to G0/1 peak ratio is as low as 1.5%.

We detected a new type of immunophenotypic abnormality associated with neoplasia, namely the observation of a discrete population of T-cells with higher than normal levels of T-cell receptor expression. In some patients abnormal T-cell receptor expression may be the only immunophenotypic evidence of neoplasia. This phenomena is also observed in adult T-cell leukemia associated with HTLV-1 infection.

We have observed abnormally high levels of gamma delta T-cells in the peripheral blood of a series of patients with non-Hodgkin's lymphoma. These normal gamma delta T-cells were CD5 negative as well as negative for both CD4 and CD8, an immunophenotype indicative of neoplasia in alpha beta T-cells. Therefore, the presence of significant numbers of gamma delta T-cells in the peripheral blood of patients with non-Hodgkin's lymphoma may lead to a false diagnosis of peripheral blood involvement with a T-cell neoplasm.

Major Findings:

1. There is good correlation among S-phase determinations made using algorithms that subtract debris based upon a model of nuclear slicing or fragmentation. However, S-phase estimates made using algorithms that subtract debris based upon an exponentially decreasing power function were discordant.

2. S-phase estimates are affected by initial placement of debris boundaries, the coefficient of variation of the G1/0 peak, and the relative amount of debris.

3. Debris compensation is necessary in diploid DNA histograms where the debris is as low as 1.5%.

4. Peripheral blood involvement with mycosis fungoides and adult T-cell leukemia associated with HTLV-1 can be detected flow cytometrically based upon levels of T-cell receptor expression.

5. Patients with non-Hodgkin's lymphoma may have increased numbers of normal gamma delta T-cells in the peripheral blood resulting in a erroneous diagnosis of peripheral blood involvement with a T-cell neoplasm unless the immunophenotypic panel includes antibodies to detect gamma delta T-cells.

Publications:

Kuchnio M, Sausville EA, Jaffe ES, Greiner T, Foss FM, McClanahan J, Fukushima P, Stetler-Stevenson M. Flow cytometric detection of neoplastic T cells in patients with mycosis fungoides based upon levels of T-cell receptor expression. Am J Clin Pathol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09373-03 LP

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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2.50	2.50	

CHECK APPROPRIATE BOX(ES)

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

B

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

We have studied the expression of TIMP-1 in Burkitt's lymphoma. TIMP-1 is expressed by 4 out of eight Burkitt cell lines studied. In vitro studies indicate that the cell lines expressing TIMP-1 have a higher invasion potential. In the nude mouse, TIMP-1 expressing cell lines are more tumorigenic and invasive. The TIMP-1 negative cell lines are not invasive and have a low incidence of tumor formation. The TIMP-1 expressing cell lines are resistant to cold shock induced apoptosis while the TIMP-1 negative cell lines readily undergo apoptosis. In addition, all of the cell lines negative for TIMP-1 expressed surface CD10 while all cell lines positive for TIMP-1 failed to express CD10. CD10 is the neutral endopeptidase 24.11, a cell-surface zinc metalloproteinase that may be involved in regulation of B-cell ontology. We have prepared a retroviral construct containing TIMP-1 and are currently infecting TIMP-1 negative cell lines in order to study the effect of TIMP-1 on growth rate, invasiveness, apoptosis, and CD10 expression.

Major Findings:

1. TIMP-1 expression in Burkitt's lymphoma cell lines is associated with increased invasion potential in vitro and in vivo.

2. TIMP-1 expression in Burkitt's lymphoma cell lines is associated with resistance to cold shock induced apoptosis.

3. TIMP-1 expression correlates with cell surface expression of neutral endopeptidase 24.11.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09144-10 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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)*

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	E. Michelotti	Biotechnology Fellow	LP NCI
	G. Michelotti	Biotechnology Fellow	LP NCI
	T. Tomonaga	Visiting Fellow	LP NCI
	T. Davis-Smith	Biotechnology Fellow	LP NCI
	E. Alesse	Visiting Fellow	LP NCI

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7.5	6.2	1.3

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have been studying three regulatory elements of the human c-myc proto-oncogene and the proteins which bind to them.

1) A cell type and differentiation specific positive cis-element, FUSE, resides 1.5 kb upstream of promoter P1. A sequence specific, single strand DNA binding protein, FUSE binding protein, FBP, interacts with this element. Cloning of FBP revealed a protein comprised of alternating amphipathic helices (five) and repeating units (four). FBP possesses the ability to displace oligonucleotides bearing its binding site from DNA, and hence can invade a double helix. Characterization of the structural features required for specific binding and for activation of gene expression are in progress. At least two proteins highly related to FBP have been cloned in our group. Comparison of the structure and properties of FBP and its relatives may help to define the molecular and physiological properties of this family.

2) A complex set of factors binds 100-150 bp upstream of P1 to a cytidine-rich nonanucleotide sequence, repeated five times. This region is required for transcription from P1, augments P2 mediated expression and stimulates reporter gene expression when fused to a heterologous promoter. Surprisingly, several sequence specific, single strand DNA binding proteins recognize this region. One of these proteins, binding to the pyrimidine-rich strand is hnRNP K. *In vitro* transcription experiments and transfection studies indicate that hnRNP K participates in the stimulation of gene expression through the CT-element. hnRNP K interacts with molecules in the TFIID fraction, including TBP. hnRNP K also binds to the tumor suppressor protein, retinoblastoma--Rb. Binding to the opposite strand are protein products of alternately spliced mRNAs encoding a zinc finger protein. We are accumulating evidence that this protein also interacts with the cell's transcription apparatus and molecules which regulate cell growth.

3) Approximately 1 kb downstream of the c-myc promoter P1 is an element which has been found to be frequently mutated in Burkitt lymphoma. We have purified a 140 kDa phosphoprotein which binds to this intronic cis-element. Analysis of tryptic peptides indicates that the protein is RFX1, or an extremely close relative of this DNA binding regulator of MHCII expression. Surprisingly, the site to which RFX binds in the intron of the human c-myc gene is a positive element in transient transfection assays.

The Far Upstream Element (FUSE) of the human c-myc proto-oncogene stimulates expression in undifferentiated cells. A FUSE binding protein (FBP) is present in undifferentiated but not differentiated cells. Peptide sequences from the purified protein allowed cloning of cDNAs encoding FBP. Expression of FBP mRNA declined upon differentiation, suggesting transcriptional regulation of FBP. Features in the FBP cDNA suggest that FBP is also regulated by RNA processing, translation and post-translational mechanisms. Both cellular and recombinant FBP form sequence-specific complexes with a single strand of FUSE. Transfection of FBP into human leukemia cells stimulated c-myc-promoter driven expression from a reporter plasmid in a FUSE dependent manner. Deletion and insertion mutagenesis of FBP defined a novel single strand DNA binding domain. Analysis of the primary and predicted secondary structure of the amino acid sequence reveals four copies of a reiterated unit comprised of a 30 residue direct repeat and an amphipathic alpha helix separated by an 18-21 residue spacer. The third and fourth copies of this repeat-helix unit constitute the minimum single strand DNA binding domain. To determine whether the FUSE site, *in vivo*, possesses single strand conformation, and therefore could be bound by FBP, cells were treated with potassium permanganate (KMn_{O4}) to modify unpaired bases. Modification of genomic DNA *in vivo* revealed hyper-reactivity associated with single strand DNA in the FUSE sequence and protection on the strand which binds FBP *in vitro*. The role of single strand DNA and single strand binding proteins in c-myc regulation is being examined. FBP has now been shown to possess the ability to separate strands in a sequence dependent manner; this activity is separable from DNA binding. Additional studies to probe for activation domains embedded within FBP1 are in progress. To evaluate further the significance of FBP, two highly related molecules, FBP2 and FBP3 have been cloned.

The CT element is a positively acting homopyrimidine tract upstream of the c-*myc* gene to which Sp1 and heterogeneous ribonucleoprotein particle (hnRNP) protein K have been shown to bind specifically. Immunodepletion of Sp1 only partially reduces CT-dependent transcription, suggesting the involvement of other factors. hnRNP protein K binds with high affinity to the pyrimidine-rich strand of the CT element. An oligodeoxyribonucleotide corresponding to this strand diminished CT-mediated transactivation when added directly to *in vitro* transcription reactions and removed *trans*-factors required for CT-activity when immobilized on columns. Surprisingly, NF1/CTF activation was also depleted by the affinity column. Both CT- and NF1/CTF mediated transactivation were restored to the column flowthrough by complementation with the high salt eluate. To test whether hnRNP K interacts with the RNA polymerase II machinery, as expected for a transcription factor, extracts were passed over a column coupled with recombinant hnRNP protein K. Once again, the column removed an activity(s) required for both CT and NF1/CTF transcription stimulation, and both CT and NF1/CTF regulation were restored by the salt eluate of the hnRNP protein K column. CT regulation was completely restored by the TFIID fraction, suggesting that hnRNP protein K interacts with either the TATA binding protein (TBP) or a TBP-associated factor. Indeed, hnRNP protein K bound directly to TBP. Reports that hnRNP protein K is cell cycle regulated prompted studies which indicate an interaction with the tumor suppressor protein, Rb. Finally, the demonstration of striking *in vivo* hypersensitivity bases in the CT region to the single-strand specific oxidizing agent KMn_{O4} is supportive of a functional role for hnRNP K in the regulation of c-myc transcription.

To evaluate better the molecular features of hnRNP K which allow sequence specific DNA binding, several mutant hnRNP K proteins were constructed and expressed as GST-fusion proteins. In addition, mutant CT-cis-elements were constructed to decipher the nucleic acid sequence requirements for hnRNP K binding. hnRNP K has more than one domain capable or recognizing the CT-element. This observation is nicely compatible with the observation that stable complex formation requires two CT-element repeats and that these repeats cannot be immediately adjacent to each other. Because hnRNP K was initially identified in hnRNP complexes, it was important to compare directly the DNA and RNA binding properties of hnRNP K. First, hnRNP K-CT-element complexes are far more effectively reduced when challenged with bulk single, strand DNA than with bulk RNA. Second when the CT-element is synthesized with the identical sequence as an oligo-ribonucleotide (rCT) or as an oligo-deoxyribonucleotide (dCT) and employed as a ligand for binding to hnRNP K, quantitative analysis indicates that dCT is preferred by 10 to 20-fold. In addition, bulk single strand human DNA competes for binding with dCT considerably more effectively than total RNA or double strand DNA.

Binding to the purine-rich strand of the CT-element are protein products of alternately spliced mRNAs encoding a zinc finger protein. We are accumulating evidence that this protein also interacts with the basal machinery and, significantly, also binds to hnRNP K.

1000 bp downstream of the c-myc promoter P1 is a site frequently mutated in Burkitt lymphoma. This site binds a 140 kDa nuclear protein in a phosphorylation dependent manner. The sequences of several peptides derived from the 140 kDa protein by trypsin digestion, identified the molecule as being RFX1 or highly related to RFX1. RFX1 is factor associated with the induced expression of MHC II; the binding of RFX to certain sites is known to be phosphorylation dependent. Defying initial and obvious speculation that this element should be a negative element whose inactivation would lead to augmented c-myc expression, substitution of the binding site with artificial polylinker sequence decreased expression of a c-myc-CAT reporter As recent experiments have indicated that enforced c-myc expression in the presence of an incomplete growth signal may trigger apoptosis, the notion that tumor progression might select for intermediate levels of c-myc expression should be entertained. In this scenario, the initial translocation in Burkitt cells would increase c-myc expression allowing proliferation in those sites such as lymph nodes or bone marrow replete with growth factors capable of driving B-cell proliferation. Tumor growth at distant sites, initially blocked by apoptosis, would be facilitated by secondary mutation of c-myc positive cis-elements. The balancing of c-myc levels sufficient to drive tumor growth but below the threshold necessary to trigger cell death might prove to be a key determinant of tumor growth.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09395-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Analysis of the Role of junD/AP-1 Complexes During T-cell Activation

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

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1.0	1.0	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The AP-1 transcription factors are a ubiquitously expressed family of phorbol ester inducible proteins that play critical roles in the control of several cellular responses to extracellular stimuli including cellular growth, proliferation, and differentiation. Recently, the AP-1 family of proteins have been shown to play an expanded role in the lymphoid specific expression of interleukin-2 during T-cell activation. We have identified and purified a novel junD/AP-1 containing multi-component complex that pre-exits in T-cells prior to stimulation. This complex, referred to as TAP-1 (T-cell AP-1) is composed of junD and a second novel 24-23kD component that we have term AAF-1 (AP-1 associated factor). Together this JunD/AAF-1 complex displays DNA binding stability and specificity that is distinct from junD homodimers alone. The DNA-binding properties of this complex is highly dependent on phosphorylation and biochemical analysis indicates that there are other separate pools of junD in T-cell nuclear extracts with differing affinities for AP-1 binding sites. AAF-1 is a DNA binding protein that shows specific binding to AP-1 and AP-1 like enhancer elements. Moreover, AAF-1 dependent formation of DNA-binding complexes with junD is highly protein and protein phosphorylation specific. Interestingly, we have also found that AAF-1 is able to stimulate the DNA binding activity of NF-AT and the oct-1/OAP complexes, suggesting that it may play a major role in the cyclosporine sensitive signal transduction pathways involved in T-cell function. The amino acid sequence of several proteolytic fragments of AAF-1 have been deduced and indicate that it is a novel protein(s). Antibodies raised against these peptides crossreact with a lymphoid specific 24kD polypeptide in T-cell nuclear extracts.

Major Findings:

1.) Multiple forms of *junD* exist in T-cells with differing capacity to specifically bind AP1 sites.

2.) The DNA-binding activity of junD in association with other factors is regulated by phosphorylation.

3.) AAF-1 (AP-1 associated factor one) is a novel DNA binding protein that enhances the DNA binding activity and alters the DNA specificity of *junD/AP1* containing complexes. This interaction is specific for *junD* since it inhibits rather than stimulates *c-jun* binding. In addition AAF-1 is dependent on phosphorylation of *junD* for complex formation.

4.) AAF-1 can activate transcription from AP-1 enhancers elements in vitro.

5.) AAF-1 can stimulate the binding of other AP-1 containing transcription factor complexes important in T-cell activation, including NF-AT and Oct-1/octamer associated protein.

6.) Microsequence analysis of tryptic peptides derived from AAF-1 indicate it is a novel protein.

7.) Preliminary immunological studies with antibodies raised against synthetic AAF-1 polypeptides indicates that AAF-1 is lymphoid specific.

Prior studies in the section have identified an AP-1 consensus sequence within the 22 bp principal enhancer element of the gibbon ape leukemia virus, (a member of the family of type C retroviruses associated with several distinct hematopoietic malignancies). Subsequent studies have shown that the gibbon T-cell lymphoma cell line, MLA 144 contains factors that strongly transactivated this AP-1 enhancer element (GALV-AP1) *in vivo*.

Efforts to isolate these factors have led to the identification of a novel multicomponent transcription factor complex (T-AP1) that pre-exists in resting T-cells. T-AP1 is induced during T-cell activation by the addition of phorbol esters. This induction occurs in the absence of protein synthesis, and therefore suggests that T-AP1 may play a significant role in the earliest stages of T-cell activation through rapid modulation of its function by post-translation modification.

The T-AP1 complex has been purified over 9000 fold from MLA 144 cells and has been found to be formed from multiple components. One component is comprised of *junD* 43kD and 38 kD polypeptides. A second component, identified by it partial dissociation from T-AP1 during purification, has been purified. This component, termed AAF-1 (AP-1 associated factor one) is comprised of multiple low molecular weight polypeptides between 27-23 kD with two major polypeptides of 24 and 23 kD. AAF-1 is able to stimulate purified *junD* DNA-binding over 100 fold, and can increase AP-1 enhancer directed transcription *in vitro*. The function of AAF-1 is highly specific for *junD* since it inhibits rather than stimulates *c-jun* DNA

binding activity *in vitro*. The DNA-binding activity of the T-AP1 complex is blocked by acid phosphatase, implicating phosphorylation as a necessary post-translational modification for function. Order of addition phosphatase experiments suggests a predominant role for the phosphorylation of junD in the formation of the TAP-1 complex. This observation is further underscored by the reduced responsiveness of recombinant *junD* to purified activator. Identification of the site of this phosphorylation and its role in modulating *junD* function is currently under investigation.

JunD and AAF-1 constitute the essential elements of the T-AP1 complex since gel purified and renatured *junD* 43 or 38 kD polypeptides in combination with either of the renatured AAF-1 polypeptides (27-23 kD) can reconstitute the TAP-1 complex. Numerous properties of purified T-AP1 distinguish it from junD. T-AP1 binds DNA with a 10 fold higher affinity, has a 90 fold longer half-life, makes twice as many contacts with the flanking DNA sequences contained within the GALV-AP1 site, and shows different relative DNA specificity by comparison to recombinant junD. The interaction of AAF-1 with *junD* and DNA is highly protein specific since AAF-1 inhibits rather than stimulates the DNA binding activity of c-*jun* homodimers.

Interestingly, AAF-1 has also been found to modulate the binding of other AP-1 containing complexes involved in T-cell activation. One these complexes, NF-AT (nuclear factor of activated T-cells) plays a major role in the induction and T-cell restricted expression of IL-2 during T-cell activation. NF-AT has been found to be composed of a cyclosporine A sensitive component variably complexed with different AP-1 family members, including *junD, c-jun, c-fos, and FosB.* AAF-1 is able to stimulate over 50 fold the binding of NF-AT to its cognate binding site in the IL-2 promoter and also stimulates transcription from a NF-AT enhancer driven promoter *in vitro*.

A second important enhancer element in the IL-2 promoter is NF-IL2A. This site has been shown to be transactivated by the oct/OAP (octamer/octamer associated protein) complex which has recently been found to contain *junD* as a major constituent. Preliminary experiments indicate that activator is able to stimulate the DNA binding of this complex at least 5-10 fold.

It appears that the activator may have a general function as a adaptor protein that augments and stabilizes interactions between *junD*/AP1 containing complexes and specific enhancer elements in T-cells. The IL-2 promoter also contains functional AP-1 sites. Therefore, as a positive modulator of 3 distinct *junD*/AP1 containing complexes that are highly active at 3 distinct enhancers within the IL-2 promoter, the activator appears poised to assume a major role in T-cell activation.

Evaluation of tryptic peptide sequences derived from purified AAF-1 suggests that AAF-1 is a unique protein. Antisera raised against these peptides cross-react with a lymphoid specific 24 kD polypeptide in T-cell nuclear extracts.

During the purification of TAP-1, it was found that there are other populations of *junD* in T-cell nuclear extracts that, through either post-translational modification or association with other factors, are incompetent to bind AP-1 binding sites. Preliminary studies using both immunoprecipitation of 35S

metabolically labelled cells with anti-*junD* antibodies and *junD* affinity chromatography indicates that a separate class of *junD* molecules is associated with multiple protein factors (18 kd, 20 kD 60kD and 90 kD) that do not co-purify with junD by DNA affinity purification and may therefore modify the DNA-binding and/or other functions of *junD*. The identities of these factors are currently under investigation.

Understanding of AAF-1 and the role of differential modification of junD during T-cell activation will provide greater insights into the control and possible pharmacological manipulation of lymphoid cellular proliferation and differentiation in both the normal and diseased state. Such an understanding could lead to the development of additional molecular strategies for the diagnosis, treatment and prevention of lymphoid cancers in addition to numerous immune diseases.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09396-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

In Vivo Pharmacological Modulation of HIV Expression by Calcium Response Modifiers

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1.0	1.0	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The transition from persistent to lytic infection by the human immunodeficiency virus is marked by a burst of viral replication and gene expression that is linked to the antigen induced immune activation of HIV-infected cells. This process occurs through the transcriptional activation of viral genes by the interaction of both cellular and virally derived factors with numerous regulatory *cis*-elements present in the HIV-1 long terminal repeat. The overlapping requirement between T-cell lymphokine genes such as interleukin 2 and the HIV-LTR for specific cellular factors forges the inextricable link between T-cell activation and activation of the HIV-LTR. We have found that prior treatment of human T-cells in culture with a calcium response modifier, carboxyamido-triazole, CAI (an oral drug currently in phase I clinical trials as an antimetastatic agent), completely inhibits mitogen stimulated transcription from the HIV-LTR. This effect is specific for the HIV-LTR as other mitogen activated transcription factors such as AP-1 are not affected by the drug. Biochemical analysis of other T-cell transcription factors important in T-cell activation show that CAI greatly inhibits both *in vivo* and *in vitro* function of the calcium dependent trans-acting factors such as NF-AT. Preliminary biochemical characterization of DNA binding activity in nuclear extracts from T-cells activated in the presence or absence of CAI shows complete inhibition of NF-AT DNA binding activity with partial inhibition of factors that bind the NF-kB motifs within the HIV-LTR. No effect was observed on AP-1 DNA binding activity. Interestingly, a novel site upstream of the kB motifs binds a previously unrecognized factor that is induced over 30 fold in activated T-cells and completely inhibited by CAI. The possible role of this site and the factor that bind it are under investigation. Current efforts are directed at identifying the elements within the HIV-LTR that are critical for CAI mediated inhibition of activated transcription from the HIV-LTR.

Major Findings:

1.) CAI is a potent inhibitor of activated transcription from the HIV-LTR.

2.) CAI also inhibits NF-AT driven transcription and therefore has potential use as an oral immunosuppressant.

3.) T-cells contains a complex that binds upstream elements in the HIV-LTR that is induced 30 fold in activated T-cells, but is nearly completely abrogated by treatment with CAI.

CAI is a carboxyamido-triazole that is currently under investigation for its efficacy as a calcium response modifier in the treatment of metastatic ovarian cancer. The mode of action of this drug are not clearly defined, but it appears to be a potent inhibitor of increases in intracellular calcium. Since calcium is known to be a major second messenger during T-cell activation, it was postulated that this drug may have some use in dissecting some of signal transduction pathways that are critical in T-cell activation. Because of the linkage of activation of the HIV-LTR to immune activation of T-lymphocytes it was also postulated that, like cyclosporin A, effects seen by this drug may have importance in modulating the transcriptional activation of HIV. Pre-incubation of jurkat T-cell in culture with CAI leads to a near complete inhibition of activation of the NF-AT (Nuclear factor of activated T-cells) driven reporter contructs in transfection assays while having no effect on mitogen activated transcription from an AP-1 driven promoter. Examination of DNA binding activity in nuclear extracts from Jurkat cell activated in the presence of CAI showed that, while AP-1 DNA binding activity was unaffected, there was complete inhibition of NF-AT DNA binding complexes. Since NF-AT function is critical for appropriate T-cell restricted expression of interleukin 2. It is postulated that like cyclosporine, CAI may have utility as an oral immunosuppressant drug at appropriate doses.

This observation was extended by analogy to the activated transcription from the HIV-LTR. As for cyclosporine A, CAI was very effective in causing near complete inhibition of transcriptional expression from the HIV-LTR in transfection assays. Analysis of nuclear extracts for factors that bound enhancer elements within the HIV-LTR showed a 50% reduction in binding activity specific for the HIV-kB sequences. In addition a previously unrecognized factor that binds a sequence upstream of the kB motifs and is induced 30 fold in activated T-cells was nearly completely inhibited.

This complex is specific since it is not competed by DNA containing either AP-1, NF-AT of NF-kB sites. U.V. crosslinking analysis suggests that it contains multiple components with molecular weights of 24 kD, 46 and 70kD. Preliminary immunological experiments suggests that the complex may contain AP-1 or AP-1/like constituents although the complex is not competed by DNA's containing a perfect AP-1 consensus sequence. The significance of this site and the factors that bind to it during T-cell activation are currently under evaluation. Currently, studies are directed at delineating the cis acting elements within the HIV-LTR and the biochemical targets that are responsible for CAI induced inhibition of HIV transcription. In addition, efforts are underway to assess the possible potency of CAI as an immunosuppressant.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09357-04 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Molecular Immune Activation Section	LP NCI
OTHER:	P. Davis	Technician	LP NCI
	T. Santoro	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Molecular Immune Activation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.4	1.4	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Mitogenic stimulation of quiescent cells results in the expression of new gene products that play a role in monitoring the extracellular environment relative to the progression of cells through the cell cycle. We have cloned a mitogen-induced gene from T cells, GEM, that encodes a GTP-binding protein that is a novel member of the ras family. Ras proteins and their relatives function as regulatory binary switches in the cell, cycling between active and inactive states. Several lines of evidence suggest that GEM may play a role in signal transduction during G1 progression. GEM protein is transiently expressed in mid-G1 between 4 and 8 hours after activation. GEM is associated with the inner face of the plasma membrane and is phosphorylated on tyrosine. Constitutive overexpression of GEM is potently anti-proliferative in normal and transformed 3T3 cells. Characterization of the mechanism reponsible for GEM-mediated growth inhibition and identification of the signal transduction pathway modulated by GEM expression may reveal novel protein associations and cell cycle controls.

Major Findings:

A gene has been isolated from an activated human T cell cDNA library that encodes a novel 34 kD GTP-binding protein designated GEM (GTP binding and Mitogen induced). The protein sequence of GEM is related to the ras super family of small GTP-binding proteins with homology extending beyond the motifs involved in GTP binding. GEM exhibits the strongest homology (37% identity) with the Dictyostelium discoideum transforming protein DdrasG. GEM conserves the GXXXXGK concensus element (residues 82-88) which is believed to be important in binding the α and β phospates of GTP, and the NKXD (residues 191-194) and EXSAX elements (residues 219-223) which confer guanine nucleotide specificity. GEM does not conserve the DXXG motif which is involved in binding and hydrolysis of the γ phosphate of GTP, substituting a glutamic acid (residue 134) for the invariant aspartic acid. A comparison of the primary structures suggests that GEM and DdrasG are members of distinct families of G-proteins. A protein related to GEM, designated RAD, has recently been described. RAD is over-expressed by ten-fold in skeletal muscle from type II diabetes as compared to normal skeletal muscle. GEM and RAD share approximately 60% homology, and both contain the unusual EXXG motif. We have shown that GEM binds GTP in whole cell extracts by immunoprecipitating GEM following ultaraviolet photoaffinity labeling.

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 most highly in spleen, thymus, kidney, and lung tissue. We have shown that transfected and endogenously expressed GEM localizes to the inner face of the plasma membrane, and to vesicles surrounding the nucleus. The carboxy but not amino terminus is required for membrane localization, although the mechanism of localization is not through a known isoprenylation concensus sequence. GEM protein is highly phosphorylated, including tyrosine phosphorylation. 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

As one approach to investigate the biological role of GEM, we have transfected GEM under the control of an MMTV promotor into NIH 3T3 cells. GEM expression was found to be stringently anti-proliferative such that it was not possible to select permanently transfected clones. GEM was equally growth inhibitory in 3T3 cells transformed by v-fms, v-ras, or v-raf. Since v-raf signalling is downstream of ras activation, the anti-proliferative activity of GEM in v-raf transformed cells suggests that GEM does not act through an inhibitory effect on ras-mediated signal transduction. Five independent clones were isolated from several million NIH 3T3 cells that had been transfected with an MMTV-GEM expression vector. Analyses of these rare clones showed that GEM RNA or protein was not expressed, and therefore, there is a complete correlation between constitutive GEM expression and growth inhibition.

The anti-proliferative effect of constitutive GEM expression is interesting in another context. Jurkat T cells do not express GEM constitutively nor after antigen or mitogen activation. Jurkat cells, a leukemia-derived cell line of relatively mature T cell phenotype is frequently used as a model to define the signal transduction pathways eminating from cross-linking the T cell receptor. GEM is unique compared to several other mitogen-induced genes isolated from peripheral blood T cells that mimic normal T cell activation in Jurkat cells by showing inducible expression. GEM expression is likewise not inducible in the murine leukemia cell line, EL4. The GEM genomic locus is not deleted or rearranged in Jurkat cells. An interesting possibility is that GEM expression has been selected against during the transformation process in Jurkat. Regulation of GEM expression may utilize a unique signal transduction pathway relative to those that have been defined for several other mitogen-induced genes in Jurkat cells.

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 future genetic manipulations such as knockouts) and to isolate the regulatory region of this gene. The GEM coding sequence is contained on 5 exons. GEM is located on chromosome 8 (8;q22-q24).

Publications:

Maguire JE, Santoro T, Jensen P, Yewdell J, Siebenlist U, Kelly K. GEM: An immediate early protein belonging to the Ras family. Science (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09358-04 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Chief, Molecular Immune Activation Section	LP NCI
OTHER:	P. Rohan	Biotechnology Fellow	LP NCI
	P. Jensen	Technician	LP NCI
	Y. Ward	Fogarty Fellow	LP NCI

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Molecular Immune Activation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.4	2.4	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Mitogenic stimulation of cells induces rapid and transient activation of MAP kinases. Previously we have identified PAC1 as an immediate early, mitogen-inducible, tyrosine phosphatase in nuclei of T cells. Recently, we have shown that PAC1 is a dual specificity phosphatase that dephosphorylates and inactivates MAP kinase in vitro and in vivo. PAC1 RNA and protein are short-lived, and it appears that controlling the transcription of PAC1 is the predominant form of its regulation. Thus, the induction of an early response gene, PAC1, results in the feed back attenuation of a primary signalling pathway.

PAC1 expression is limited to hematopoietic cells. PAC1 is related to a serum-inducible gene, 3CH134, that has recently been shown to encode a MAP kinase phosphatase expressed in non-hematopoietic cells. In addition to PAC1 and 3CH134, biochemical and genetic approaches suggest that other genes closely related to PAC1 exist. The potential differential regulation of different MAP kinase phosphatases suggests an unforeseen level of kinase regulation. It is becoming apparent that there are many enzymes structurally related to the MAP kinases, some of which are part of distinct signal transduction pathways. An important question is the relative biological role of the MAP kinase phosphatases, at least partly defined by their substrate specificity, tissue distribution, and regulation.

Major Findings:

We have cloned a mitogen-induced gene from human peripheral blood T cells that encodes a nuclear protein phosphotyrosine phosphatase (PTPase), designated PAC1 for phosphatase of activated cells. Several lines of evidence indicate that PAC1 is a physiologically relevant MAP kinase phosphatase. Recombinant PAC1 in vitro is a dual-specific Thr/Tyr phosphatase with stringent substrate specificity for MAP kinase. Several other tyrosine or threonine phosphorylated peptide substrates were not dephosphorylated by PAC1. Constitutive expression of PAC1 in transiently-transfected COS cells led to selective inhibition of MAPK phosphorylation and activity normally stimulated by EGF or PMA. Permanently-transfected Jurkat T cell clones expressing relatively low levels of PAC1 demonstrated substantially reduced MAP kinase activity following T cell receptor cross-linking.

PAC1 is a nuclear phosphatase that is likely to act upon MAP kinases that are translocated into the nucleus following activation. In order to examine the effect of PAC1 expression on a nuclear event linked in vivo to MAP kinase activation, we utilized a transient transfection assay that measures the transcriptional activity of the c-fos serum response element (SRE). In transiently-transfected CV-1 cells, increases in SRE activity resulting from PMA treatment or from increased ERK2 expression and activation are inhibited in vivo by PAC1. Although the specific nuclear events that act upon the SRE are not directly addressed by these experiments, a PMA-stimulated, MAP kinase-mediated pathway involving the SRF/Elk-1 ternary complex has been described.

MAP kinase activation is regulated in mature T cells by the same signals that induce PAC1 expression. The dephosphorylation and inactivation of MAP kinase following signal transduction correlates approximately with the accumulation of PAC1 in T cells between 30 and 60 minutes after receptor cross-linking. PAC1 RNA and protein are short-lived, and it appears that controlling the transcription of PAC1 is a predominant form of regulation.

The conserved catalytic site of tyrosine phosphatases has been defined and found to involve an essential cysteine (Cys-257 in PAC1). We have observed that catalytically-inactive recombinant PAC1 in which Cys-257 has been substituted by Ser binds ERK2 from cell extracts while no such binding is evident with wild type PAC1. ERK2 activity is inhibited by binding to PAC1-Ser-257. Thus, PAC1-Ser-257 might be expected to act as a dominant negative form of PAC1.

It seems highly likely that MAP kinase activity would be essential for normal growth, although this has not been directly tested with knockouts. Consistent with the essential nature of MAP kinase, we have been unable to select 3T3 cells transfected with a vector in which PAC1 expression is regulated by the MMTV LTR. PAC1-Ser-257 expression constructs gave identical results to those of the wild type PAC1, suggesting a dominant negative phenotype relative to MAP kinase activity.

PAC1 seems to be a member of a family of related phosphatases. 3CH134 is a mitogen-regulated MAP kinase phosphatase that is expressed in non-hematopoietic tissues. In addition, several experimental lines of evidence suggest that there are additional phosphatases related to PAC1 and 3CH134. 1) We have observed several cross-hybridizing bands at low stringency and 1 additional band to PAC1 at high stringency in genomic DNA using 5' and 3' coding probes from PAC1. No such hybridization is observed with a 3' untranslated region probe, indicating that cross-hybridizations most likely do not represent pseudogenes. 2) We have observed a 39 kDalton mitogen-induced protein in T cells that is highly related to PAC1 as evidenced by its binding with several monoclonal and anti-peptide PAC1 antibodies. 3) Northern blots reveal PAC1 cross-reactive messages of distinct sizes and tissue distribution. PAC1 is expressed at high levels in spleen and thymus, low levels in bone marrow and is not expressed in fetal liver. There is a cross-reactive message that is highly expressed in fetal liver and bone marrow, but not expressed in thymus or spleen, consistent with a tissue specificity of hematopoietic progenitor cells.

Publications:

Ward Y, Gupta S, Jensen P, Wartmann M, Davis RJ, Kelly K. Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. Nature 1994;367:651-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09389-01 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

A Mitogen-induced G Protein-coupled Receptor

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

PI:	K. Kelly	Chief, Molecular Immune Activation Section	LP NCI
OTHER:	J. Gray	Fogarty Fellow	LP NCI

COOPERATING UNITS *(if any)*

Hematopathology (Drs. Mark Roth, Maryalice Stetler-Stevenson, and Elaine Jaffe)

LAB/BRANCH

Laboratory of Pathology

SECTION

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NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. A mitogen-induced gene cloned from T cells, 276, encodes a protein of approximately 87 kD. 276 is constitutively expressed on neutrophils and macrophages and is induced in its expression on T cells and B cells. The carboxy half of the protein demonstrates the seven membrane spanning conserved structure of the class of receptors that bind heterotrimeric G proteins, and a large extracellular domain contains EGF-like repeats and an RGD sequence. The structure of the extracellular domain suggests a role in cell/cell or cell/matrix interactions. The heptahelical structure implies a signal transducing function. The cellular distribution of 276 suggests that it may be involved in a function (possibly adhesion) mediated at inflammatory sites.

Major Findings:

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 a 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. The 276 protein migrates unexpectedly fast on SDS-PAGE. Pulse-chase experiments demonstrated processing of the 87 kD peptide backbone structure. 276 was processed in transfected COS-7 cells to generate an N-linked deglycosylated peptide that migrates at 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. Whether this unpredicted migration is due to post-translational modification or to an unusual assumed conformation is being investigated.

In order to better understand the physiological role of 276, we have investigated its expression profile. FACS analyses showed expression of 276 in neutrophils, monocytes, and activated T and B cells. Immunohistochemical analyses of normal lymph node has demonstrated expression in histiocytes and a subpopulation of T and B cells. In addition, several T cell lymphomas but not B cell lymphomas are positive for 276 expression. 276 expression in cells at inflammatory sites is being determined.

We have produced a soluble form of the extracellular domain in order to assay its interaction with extracellular matrix or other cell surface-associated structures. In addition, such a soluble molecule will be useful as a competitive blocking agent.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09170-07 LP

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Genes Regulating Pattern Formation During Embryonic Development

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

PI:	S. Mackem	Expert	LP NCI
OTHER:	R. DeSanto	General Fellow	LP NCI
	V. Knezevic	Visiting Fellow	LP NCI
	N. Ljubic	Visiting Fellow	LP NCI

COOPERATING UNITS *(if any)* K. Mahon, LMGD, NICHD; M. Kuehn, Exp. Immunol. Branch, NCI; 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; R. Behringer, M.D. Anderson Cancer Center, Houston, Texas

LAB/BRANCH

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4	4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The identification of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. Many key processes in morphogenesis, including responses to trophic stimuli, cell-cell interactions, migration, differential cell multiplication, and programmed cell death, are recapitulated in a pathologic manner during oncogenesis and metastasis. The chick embryo is attractive for studying the molecular basis of pattern formation because of accessability of the embryo to manipulation, amenability to biochemical and molecular analyses, and a high degree of conservation with mammalian development. Hence, genes isolated in the chick can be analyzed using molecular genetic approaches in the mouse embryo, as well.

The aim of this project is to isolate developmental control genes that regulate pattern formation. Currently, two novel members of the homeobox gene family have been identified which are expressed predominantly in the limb bud during organogenesis; one of these genes is also expressed during gastrulation. These genes each show spatially restricted expression domains within the limb bud that suggest roles in regulating pattern formation along the anterior-posterior (A-P) or along the proximodistal (P-D) axes of the limb. The homeobox gene expressed during gastrulation may regulate the determination of positional identity along the primary embryonic axis, as well as the limb P-D axis. We have also identified two new members of another type of developmental transcriptional regulator, the mouse T gene (*brachyury*), which are expressed during gastrulation. This is the first demonstration that *brachyury* represents a new family of developmental regulators. Studies are underway to elucidate the function of these genes, using both molecular-genetic and biochemical approaches in the chick and mouse embryos.

Major Findings:

Oriented cDNA libraries have been generated from chick 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, or leg and late (stage 21/22) wing, or leg libraries. cDNA libraries from gastrulation-stage chick embryos have also been generated.

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* (*Gnot1*, previously *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 *Gnot1* 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 *Gnot1* in the determination of positional identity (for example of the wrist/ankle bony patterns) along the P-D axis. Microsurgical manipulations (apical ridge excisions and polarizing grafts) indicate that *Gnot1* is expressed in a position- and fate-dependent manner that changes when the developmental program (pattern) is experimentally altered. Our results indicate that the presence of the apical ectodermal ridge is absolutely required for the early *Gnot1* 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 *Gnot1* expression. Likewise, polarizing grafts that induce mirror-image duplications including the 'wrist' region, are preceeded by a mirror-image duplicated zone of *Gnot1* expression. These findings are consistent with a role for *Gnot1* in the determination of positional identity in the presumptive wrist/ankle regions.

Interestingly, *Gnot1* 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 *Gnot1* within the notochord recedes posteriorly concommitant with the adjacent segmentation of paraxial mesoderm into discrete somites. Following somitogenesis, *Gnot1* 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 *Gnot1* 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 *Gnot1* in chick embryos using retroviral expression vectors. To study effects of altering the very early expression of *Gnot1* 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.

To analyze the effects of altering *Gnot1* expression during gastrulation at the molecular level, we plan to analyze the expression of other developmental control genes expressed in 'organizer' tissues during gastrulation, such as *goosecoid* (an organizer homeobox gene) and *brachyury* (mouse T gene). In the course of isolating a chick *brachyury* gene for these studies, we discovered the existence of *brachyury*-related genes that are expressed in partly overlapping domains (relative to *brachyury*) in organizer regions during gastrulation. In the chick embryo, there are at least two such related genes. This is the first demonstration that *brachyury* is a member of a new developmental control gene family, the members of which appear to play key roles in patterning the embryonic A-P axis during gastrulation.

Publications:

Mackem S, Mahon K. Ghox 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. Limb development and regeneration. New York: Wiley-Liss, 1993;21-30.

Annual Report

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY
FY 1994

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 two carcinoma associated antigens: (a) TAG-72 (Tumor Associated Glycoprotein) recognized by MAbs B72.3 and CC49 among others, and (b) carcinoembryonic antigen (CEA) recognized by the COL MAbs. 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 monoclonal antibodies (MAbs) to two carcinoma associated antigens and the potential use of these MAbs in both the diagnosis and therapy of a range of carcinomas. These 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, (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 (177Lu)-labeled CC49 was analyzed in this same experimental model. 177Lu 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. 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 131I-COL-1 could efficiently target and eliminate the growth of established human tumors in a xenograft model. (IV) Numerous collaborative Phase I and II clinical trials with MAbs CC49,and COL-1. Studies are also in progress on the use of radiolabeled CC49 with an intraoperative hand held probe to more efficiently detect carcinoma metastases.

RECOMBINANT IMMUNOGLOBULIN WORKING GROUP: Novel Recombinant Immunoglobulin Molecules for Cancer Therapy and Diagnosis. (S. Kashmiri)

Several murine monoclonal antibodies (MAbs) with selective reactivity to carcinoma associated antigens have been developed in this laboratory. These include MAbs against carcinoembryonic antigen (CEA), tumor associated glycoprotein (TAG)-72 and a 48 kD antigen expressed on the surface of normal and malignant gastrointestinal epithelium. While some of these MAbs have shown excellent tumor targeting in ongoing clinical trials, their use for in vivo diagnosis and therapy is limited because of their immunogenicity in patients. This project is aimed toward developing novel immunological reagents for the diagnosis and therapy of human cancers,

using genetic engineering techniques. To reduce the potential problem of the immunogenicity of the anti-carcinoma antibodies, we earlier developed mouse-human chimeric (c) MAbs, including cB72.3, cCC49, cCC83 and cD612. To further reduce their immunogenicity in patients, one of our goals is to "humanize" MAb CC49. To that end, we wish to develop a well refined, high resolution, three dimensional structure of the antigen binding region of the antibody. In collaboration with Dr. Eduardo Padlan at NIH, we have carried out crystallographic studies and developed a primary structure of the anti-tumor MAb CC49 Fab'. In an effort to optimize the pharmacokinetics of plasma clearance, we developed novel chimeric immunoglobulin variants. We earlier expressed, in E. coli, a single chain antigen binding protein, sFv, derived from MAb CC49. Using a vector which carries the tac promoter and omp A signal, we have examined the effects of four variables on expression and accumulation of soluble CC49 sFv. Using retroviral expression constructs of the murine heavy and light chain genes of the anti-human colon carcinoma MAb D612, we engineered human colon tumor cells that constitutively expressed the D612-reactive antigen for the secretion of the D612 MAb. The antibody produced by these cells elicited ADCC mediated by NK cells against themselves as well as against the unmodified parental cells.

In our ongoing studies on recombinant baculovirus carcinoembryonic antigen, we have carried out serological and biochemical characterization of the recombinant protein. These studies demonstrated a virtual immunologic identity between the native and the recombinant molecules. The baculovirus recombinant CEA appeared to be a more potent humoral immunogen in mice than native CEA.

CYTOKINE WORKING GROUP: Cytokine-based Modalities to Enhance Antibody and Cell-Mediated Tumor Cell Killing. (J. Greiner)

The ability of different differentiation-modulating agents which enhance the expression of major histocompatibility as well as non-major histocompatibility antigens may be an important component in immunotherapeutic protocols based on tumor recognition by monoclonal antibodies (MAb) or antigen-driven cytotoxic lymphoid cells. Our studies have clearly shown that two cytokines, interferon-γ and interleukin-6 (IL-6), can increase HLA expression as well as selective tumor-associated antigen, such as CEA and TAG-72, on the surface of human carcinoma cells. In another study, a novel 110kD antigen was identified in human gastric carcinoma cells. Initially, its physicochemical characteristics as well as the ability of IFN- γ to upregulate its expression were indications of a link to antigen of the CEA gene family. Upon molecular cloning of the 110 kD antigen, it was found to be a unique glycoprotein not related to the CEA gene family, but, it also represents a potential novel IFN-γ regulated gene product. In the case of TAG-72, CEA and the 110 kD antigen, the increase in their expression as a result of IFN-γ or IL-6 treatment is usually accompanied by an increase in the respective mRNA transcripts. That observation indicates that new antigen synthesis is one of the components contributing to the enhanced antigen expression. The enhanced tumor antigen expression has been documented in vivo tumor xenograft model systems and has resulted in improved MAb tumor targeting as well as an augmentation in MAb-based immunotherapy. In recent experiments, a combination of IFN-γ and IL-6 showed synergistic activity with respect to their abilities to enhance CEA and HLA expression. Substantial enhancement of both surface antigens was achieved with minimal change in cell proliferation. Administration of certain cytokines or other differentiation-inducing agents can augment the level of expression of major histocompatibility and non-major histocompatibility antigens. The ability to increase human tumor antigen expression has been shown to improve radioimmunodetection and radioimmunotherapy in MAb-based experimental model systems. The cytokine-driven augmentation of class I and II HLA antigens may also play a critical in the recognition of tumor cells by cytotoxic lymphoid cells.

RECOMBINANT VACCINE WORKING GROUP: Recombinant Vaccines for Active Specific Immunotherapy of Human Carcinoma. (J. Kantor)

Certain tumor associated antigens(TAAs) represent potential targets for specific active immunotherapy. Our laboratory has been evaluating the safety, toxicity and immunogenicity of using recombinant vaccinia viruses, expressing human tumor associated antigens, as immunogens for the treatment of human carcinomas. We have constructed, characterized and determined the safety and immunogenicity of two recombinant vaccinia viruses in murine tumor models as well as rhesus monkeys. The first recombinant vaccinia virus expressed human carcinoembryonic antigen(CEA) and the second vaccinia virus expressed human prostate specific antigen(PSA). CEA is a 180Kd glycoprotein which is overexpressed in human colorectal, gastric, pancreatic, breast and non small cell lung carcinoma. PSA is a glycoprotein of 30-33 Kd which is expressed in prostrate carcinoma. It is unclear whether these TAAs are immunogenic in humans. Humoral and cell mediated responses to these tumor antigens have not been well documented in normal or cancer patients. The presentation of these TAAs in the context of Class I MHC might increase their immunogenicity and represent a logical approach to inducing anti CEA and PSA responses for tumor immunotherapy. We have recently shown that CEA could be used as a target for active immunotherapy. Anti-tumor activity was demonstrated in an animal tumor model by immunization of mice with the recombinant vaccinia virus expressing human CEA. This recombinant vaccine was also shown to induce both cell-mediated and humoral CEA specific immune responses in mice and nonhuman primates. The recombinant CEA vaccine has been evaluated in a Phase I clinical trial at the NCI from May to December of 1993. Twenty six patients who had CEA expressing carcinomas entered the trial . Seven patients received Dose level I ($2X10^5$ PFU), seven patients received dose level II ($2X10^6$ PFU) and 12 patients received dose level III ($1X10^7$ PFU). These doses were given three times by skin scarification four weeks apart. These studies have shown that the recombinat CEA vaccinia virus can be safely administered to cancer patients with no toxicity and that this vaccine can serve as an immunogen for repeated exposures to a specific tumor associated antigen. Clinical and immunological evaluations for the presence of CEA specific humoral and cell mediated responses are being performed. A recombinant PSA vaccinia virus has been constructed and evaluated for its safety and immunogenicity in a murine tumor model expressing human PSA and in male rhesus monkeys. This vaccine was shown to have no toxicity in these animal models. Monkey toxicity studies have shown that blood counts, differentials and hepatic and renal chemistries remained normal throughout the study. These animals are being evaluated for PSA specific cell mediated responses.

IMMUNOLOGY WORKING GROUP: Cellular Immunotherapy of Human Carcinoma (K. Tsang)

We have investigated a potential novel approach of combining tumor -infiltrating lymphocytes and antitumor MAbs in the creation of a T cell capable of secreting antitumor Ig, in essence, creating an antitumor Ig "factory" at the tumor site. The cDNA expression construct of the chimeric D612 heavy chain and light chain gene in retroviral vectors were introduced into MOLT-4 cells and used as a model in this studies. Chimeric D612 can be expressed and secreted by human T cell line MOLT-4. The secreted Ig retained its antigen-binding properties. We have also investigated whether human T lymphocytes are able to distinguish the determinants created by point-mutated p21ras proteins from the normal ras protein. Cellular immunity to 4 synthetic peptides representing amino acids 5-17 of mutated p21 ras proteins with an exchange of normal glycine (G12) at position 12 by valine (V12), cysteine (C12) or aspartic acid (D12)was studies. Human T-cell lines from different individuals have been established by in vitro stimulation with 13-mer peptides refecting the position 12 ras point mutations. The peptide specific T cell lines were all CD3+, CD4+ and CD8- phenotypes. Induction of proliferation and secretion of IFN-γ ,IL-2 and IL-6 but not IL4 secretion were observed when the corresponding peptide was used for stimulation. Specific cytotoxic T cell activity was detected when the corresponding mutated p21ras

peptide was used to pulse the target cells, or when the target cells used were transfected with the vector carrying the corresponding point-mutated p21ras protein. We have investigated the introduction and expression of human IL-6 gene in human colorectal carcinoma cells. cDNA encoding the human IL-6 gene inserted into retroviral expression vector, was introduced into HT-29 cell human colorectal carcinoma cells by lipofection. IL-6 secreted by the transfected HT-29 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 in the transfected HT-29 cells. These results suggest another potential role for the use of IL-6 gene transfer in the immunotherapy of human cancers. We studied the effects of recombinant human M-CSF (rhM-CSF) on the ADCC activity of human monocytes. These results demonstrated that rhM-CSF significantly augment ADCC activity of human peripheral blood monocytes using MAbs to human colorectal carcinoma, suggesting a potential role for rhM-CSF in cancer immunotherapy. We have also demonstrated that human TILS can be transduced with 3'end truncated IL-2 gene and produced high amounts of IL-2 in the supernatants. These results indicate the feasibility of employing adoptive immunotherapy procotols using IL-2 transduced TILS in cancer patients. T-cell lines specific to 9-mer or 10 mer CEA peptides were established from patients with carcinoma. Further analysis of these cell lines are in progress.

Host Immune Responses to Human Carcinoma Antigens Induced by Recombinant Vaccines. (S. Abrams)

We have continued to focus on the growing area of experimental and clinical immunotherapy of human carcinoma. Central to its investigative design is the biological application of recombinant poxviruses, recombinant proteins and/or peptides as "tumor vaccines", which specifically target those cancers. Our studies emphasize a basic and fundamental understanding of the mechanisms of action and immunological impact of such vaccines in preclinical animal models and now in patient clinical trials. Much of this work has concentrated on carcinoembryonic antigen (CEA) and the point-mutated ras p21 oncogene. The over-expression of tumor-associated antigen (Ag) (i.e., CEA) and/or the neo-expression of tumor-specific epitopes (i.e., point-mutated ras p21) may represent selective or unique targets for immune recognition, particularly by T-lymphocytes which have been implicated as important elements for host defense against malignancy. Here, in a murine model, we explored and characterized distinct effector properties of host-derived T-lymphocytes reactive to mutated ras peptides. BALB/c mice (H-2d) were inoculated with a purified peptide, 13 amino acids in length, reflecting positions 5–17 of the normal (G12) or a mutated (V12) ras protein commonly found in human carcinomas. A T-cell line and clonal derivatives of that line were established to the ras(V12) peptide, which displayed an ab-TCR+, CD3+, CD4+, CD8- phenotype and expressed Ag-specific proliferation. Cytokine secretion revealed TNF, GM-CSF, IFN-γ, and/or IL-2 patterns, consistent with the CD4+ Th1 subtypes. Moreover, Ag-specific cytotoxicity was demonstrable against syngeneic lad-bearing tumor cells either incubated with exogenously bound ras(V12) peptide or transduced with the point-mutated Ki-ras(V12) oncogene. In parallel experiments, we have begun to examine the feasibility for the induction of anti-ras CD8+ cytotoxic T lymphocytes (CTL). We have identified two mutant ras(V12) sequences, 4-12 (9-mer) and 3-12 (10-mer), as potential CD8+ epitopes for murine H-2Kd and confirmed their MHC class I binding activity by a functional competition assay. We plan to explore these peptides as well as vaccinia recombinants for their in vivo CD8+ immunogenicity. Finally, we have begun to explore the safety and efficacy of a vaccinia recombinant expressing human CEA in phase I clinical trials. Here, we examined its impact on cell-mediated immunity and found evidence, in about 50% of the patients, for the augmentation of pre-existing anti-vaccinia responses. The extent of any CEA-specific immunity is unclear and still under investigation.

ONCOGENE WORKING GROUP: Expression Cloning of Genes Capable of Suppressing *ras* Transformation. (Dr. M.L. Cutler)

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 *rsu*-1, is a novel gene which specifically suppresses v-Ki-*ras* and v-Ha-*ras* transformation of fibroblasts and epithelial cells. The *rsu*-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 *rsu*-1 may physically associate with *ras* p21or ras interacting molecules and alter *Ras* signal transduction in this way. In non-transformed cells p33 *rsu*-1 is phosphorylated in response to stimuli which activate *Ras* signal transduction pathways. Phosphorylation of p33 *Rsu*-1 is dependent on activation of *Ras* in these pathways, indicting *Rsu*-1 is part of a "downstream" effector or regulators pathway. *rsu*-1 is a phylogenetically highly conserved molecule; cloning and sequencing of the human *rsu*-1 cDNA revealed that the human *rsu*-1 protein is 96% homologous to the mouse *rsu*-1. Screening of over 100 cell lines and tissue, both human and rodent, revealed that *rsu*-1 RNA expression is ubiquitous. The human *rsu*-1 gene has been localized to human chromosome 10p13, a region of chromosome 10 frequently deleted in high grade glioblastoma. Our current efforts are aimed at elucidating the mechanisms by which this molecule disrupts *ras* signal transduction in vitro and determining if the *rsu*-1 locus is disrupted in humanglioblastomas and other tumors.

ENDOCRINOLOGY WORKING GROUP: Hormones, Antihormones and Growth Factors in Mammary Development and Tumorigenesis. (Dr. B. Vonderhaar)

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 (Prl), estrogens, and progesterone, with recent work also examining how epidermal growth factor (EGF), and transforming growth factors a and b 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, Prl and glucocorticoids, EGF or TGF-α can promote full lobulo-alveolar development in vitro. High concentrations of the somatogenic hormone, growth hormone, can substitute for Prl in this system. This effect is not mediated through insulin-like growth factor I. Lobulo-alveolar development in vitro is not inhibited by TGF-β; casein synthesis is. In addition, TGF-β dramatically inhibits ductal outgrowth by epithelial cells transduced with the human TGF-β1 gene and transplanted back into the cleared mammary fat pad. Prl induced growth of the mouse mammary epithelial cell, NOG-8, involves activation of protein kinase C (PKC). Prl 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 Prl when grown in the presence of charcoal stripped serum. This effect is reversed by the addition of Prl to the culture medium, or by introducing the *rsp*-1 *ras* suppressor gene. Finally, we have explored the relationship of a membrane associated

antilactogen binding site (ALBS) to the Prl receptor on human breast cancer cell growth in culture. Prl 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 Prl receptor. These studies are greatly aided by use of monoclonal antibody B6.2 which is the first known such antibody to recognize the human Prl receptor. The antiprolactin action of tamoxifen, working through the ALBS, may have important clinical implications.

TUMOR GROWTH FACTORS SECTION: EGF Related Peptides in Breast and Colon Cancer. (Dr. D. Salomon, Chief)

Transforming growth factor a (TGFα), 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 TGFα and AR can bind to the EGF receptor (c-erb β). TGFα 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, TGFα 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 TGFα. Furthermore, overexpression of a human TGFα cDNA in these cells leads to their in vitro transformation. Addition of an anti-EGF receptor blocking antibody or an anti-TGFα neutralizing antibody can partially or completely inhibit the growth of the Ha-ras or TGFα transformed mammary cells sugesting 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 TGFα 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 TGFα 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 TGFa promoter contains a cis-acting estrogen-responsive element(s) (ERE). MCF-7 or ZR-75-1 cells were infected with a recombinant amphotropic TGFα antisense mRNA expression vector. Expression of this antisense mRNA lead to a reduction in estrogen-induced TGFα 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 80% of primary human breast tumors at a level that exceeded the level found in adjacent normal mammary epithelium.

CELLULAR BIOCHEMISTRY SECTION: The Regulatory Subunit of cAMP-Dependent Protein Kinase as a Target for Cancer Diagnosis, Chemotherapy, and Chemoprevention. (Dr. Y. S. Cho-Chung, Chief)

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-Cl-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 RIα with up-regulation of RIIβ 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 RIα and RIIβ regulatory subunits of cAMP-dependent protein kinase, have opposite roles in cell growth and differentiation; RIα being growth stimulatory while RIIβ is a growth-inhibitory and differentiation-inducing protein. As RIα 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-Cl-cAMP and RIα antisense oligodeoxynucleotide, those that effectively down-regulate RI and up-regulate RIIβ, provide new approaches toward differentiation therapy and chemoprevention of cancer. 8-Cl-cAMP is now in Phase I clinical studies at several foreign Institutes.

Mechanism of cAMP Action Growth Control and Differentiation

The striking growth inhibitory effect of 8-Cl-cAMP has been related to its selective binding and activation of protein kinase isozymes: It binds to RII 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-Cl-cAMP brought about various effects among the cell lines tested, including the suppression of oncogenes and transforming growth factor α (TGFα), and morphological changes, differentiation, and reverse transformation. Despite the appearance of markers of mature phenotype and definitive growth arrest, the 8-Cl-cAMP-treated leukemic cells exhibited no change in the cell cycle phase. 8-Cl-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-Cl-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-Cl-cAMP include a rapid nuclear translocation of RIIβ, and such translocation of RIIβ into the nucleus correlates with an increase n 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 RIIb 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 autophorylation 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 RIIβ of introduced into RIα 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.

ONCOGENETICS SECTION: Identification and Characterization of Mutations in Breast Cancer. (Dr. R. Callahan, Chief)

We have undertaken on going 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 specfic regions of the cellular genome in tumor DNA. In previous studies we have found LOH on chromosomes 1p, lq, 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 chromosome17p13 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 messured by BUDR incorporation. Furthermore, this association appears to primarily refect those tumors having a mutaion in either exon 5 (p=0.0002) or exon 6 (p=0.05). There is a body of evience 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 chromosome17.

Mammary Tumorigenesis in Inbred and Feral Mice:

The mammary tumor system has been useful both in the identification and characterization of genes involved in the development and transformation of mammary epithelium. In previous studies we identified and characterized the Notch related Int-3 gene which is frequently rearranged by MMTV in mouse mammary tumors. More recently the analysis of one of our 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 Int-6. It was found to be rearranged by MMTV in 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. It appears to be unrelated to any known gene in the GenBank. In tumors in which Int-6 is rearranged by MMTV the intergration site is within an intron and the transcriptional orientiation of the viral genome is opposite to that of the Int-6 gene. This leads to the expression of a truncated Int-6 gene product. The biological activity of the MMTV rearranged Int-6 gene is being tested. The HC11 mouse mammary epithelial cells, while maintaing 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 condons in exon 5 and the other allele contains a missense mutation in exon6. Introduction of the wild type trp53 gene into these cells suppresses their grwoth. This cell line may therefore be useful in studies aimed at the cooperative interaction of mutations in mammary carcinogenesis.

CELLULAR AND MOLECULAR PHYSIOLOGY SECTION: Cytoskeletal Proteins in Oncogenes Transformation and Human Neoplasia. (Dr. G. Prasad)

The major goal of this research project is to understand the molecular relationship of suppression of tropomyosin (TM) expression to neoplastic transformation. Earlier work carried out in this laboratory suggested that TM suppression plays a causal role in cellular transformation. Supporting evidence for this hypothesis was obtained by restoring expression of one of the two

suppressed isoforms of TM (TM1) in a v-Ki-ras-transformed cell line, DT, by means of retroviral gene transfer. Transduced cell lines of DT which express elevated levels of TM did not participate in anchorage independent growth or tumorigenesis in nude mice. Transduced TM participated in the reemergence of microfilament network to a limited degree, as evidenced by immunofluorescence and biochemical analysis. It is important to understand the molecular basis of this phenomenon before therapies for human cancers can be considered based on TM gene replacement. Normal NIH3T3 cells were also transduced with TM1 and its metabolism was studied. Results of these experiments revealed that homodimers of TM1 protein did not form stable components in the cytoskeleton in either normal or transformed cells. This may explain the partial restoration of cytoslkelton observed with DT transduced lines. cell lines DT which express both the suppressed isoforms, viz., 1 and 2 are constructed and being examined.

Earlier, we have demonstrated severe deficiencies in TM expression in human breast carcinoma cell lines. In order to investigate if the observed anomalies of TM metabolism do exist in the human breast carcinoma cell lines, an isoform specific antibody has been developed.

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 antibodies 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 05190-14 LTIB

PERIOD COVERED: October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens

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

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Experimental Oncology Section

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

TOTAL STAFF YEARS: 7.0	PROFESSIONAL: 2.7	OTHER: 4.3

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

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

Progress has been made in the characterization of monoclonal antibodies (MAbs) to two carcinoma associated antigens and the potential use of these MAbs in both the diagnosis and therapy of a range of carcinomas. These 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. (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 (177Lu)-labeled CC49 was analyzed in this same experimental model. 177Lu 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. 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 131I-COL-1 could efficiently target and eliminate the growth of established human tumors in a xenograft model. (III) Numerous collaborative Phase I and II clinical trials with MAbs CC49,and COL-1. Studies are also in progress on the use of radiolabeled CC49 with an intraoperative hand held probe to more efficiently detect carcinoma metastases.

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 two carcinoma associated antigens and their potential use in both the diagnosis and therapy of a range of carcinomas. These 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.

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_as 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 750μCi of ^{177}Lu-CC49 (250μCi/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 $F(ab')_2$ 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_L amino acid sequence of an immunoglobulin tethered to a V_H sequence by a designed peptide. The CC49 sFv was shown to be a M_r 27,000 homogeneous entity which could be efficiently radiolabeled with ^{125}I or ^{131}I. Comparative direct binding studies and competition radioimmunoassays using CC49 intact IgG, F(ab')$_2$, Fab', and sFv revealed that the monomeric CC49 Fab' and sFv have relative binding affinities 8-fold lower than the dimeric F(ab')$_2$ 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')$_2$ 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 ^{125}I-labeled Fab' and F(ab')$_2$ was not seen with ^{125}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. ^{125}I-CC49 sFv and ^{177}Lu-CC49 sFv were coinjected in mice bearing antigen-positive carcinoma xenografts. Both sFv forms showed similar tumor targeting and plasma clearance pharmacokinetics. The ^{177}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 ^{125}I-labeled sFvs for tumor detection using the intraoperative hand-held probe, and ^{131}I (or ^{123}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. ^{125}I-labeled sFv, Fab', F(ab')$_2$, 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}I, ^{125}I and ^{177}Lu-labeled MAb CC49 are now in progress in colorectal, breast, lung, and ovarian cancer patients.

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}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}I-labeled COL-1 demonstrated reduction of tumor growth rate when 300μCi of the immunoconjugate was used. Dose fractionation experiments with ^{131}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}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: ZO1 CB 09009-13 LTIB

PERIOD COVERED: October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Augmentation of Tumor Antigen Expression

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

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Experimental Oncology Section

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

TOTAL STAFF YEARS: 5.5 | PROFESSIONAL: 4.1 | OTHER: 1.3

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

A

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

The ability of different differentiation-modulating agents which enhance the expression of major histocompatibility as well as non-major histocompatibility antigens may be an important component in immunotherapeutic protocols based on tumor recognition by monoclonal antibodies (MAb) or antigen-driven cytotoxic lymphoid cells. Our studies have clearly shown that two cytokines, interferon-g and interleukin-6 (IL-6), can increase HLA expression as well as selective tumor-associated antigen, such as CEA and TAG-72, on the surface of human carcinoma cells. In another study, a novel 110kD antigen was identified in human gastric carcinoma cells. Initially, its physicochemical characteristics as well as the ability of IFN- γ to upregulate its expression were indications of a link to antigen of the CEA gene family. Upon molecular cloning of the 110 kD antigen, it was found to be a unique glycoprotein not related to the CEA gene family, but, it also represents a potential novel IFN-γ regulated gene product. In the case of TAG-72, CEA and the 110 kD antigen, the increase in their expression as a result of IFN-γ or IL-6 treatment is usually accompanied by an increase in the respective mRNA transcripts. That observation indicates that new antigen synthesis is one of the components contributing to the enhanced antigen expression. The enhanced tumor antigen expression has been documented in vivo tumor xenograft model systems and has resulted in improved MAb tumor targeting as well as an augmentation in MAb-based immunotherapy. In recent experiments, a combination of IFN-γ and IL-6 showed synergistic activity with respect to their abilities to enhance CEA and HLA expression. Substantial enhancement of both surface antigens was achieved with minimal change in cell proliferation. Administration of certain cytokines or other differentiation-inducing agents can augment the level of expression of major histocompatibility and non-major histocompatibility antigens. The ability to increase human tumor antigen expression has been shown to improve radioimmunodetection and radioimmunotherapy in MAb-based experimental model systems. The cytokine-driven augmentation of class I and II HLA antigens may also play a critical role in the recognition of tumor cells by cytotoxic lymphoid cells.

Major Findings

Previously, we identified a M_r 110,000 antigen which had some similar immunological characteristics as carcinoembryonic antigen (CEA) and other members of that gene family. Monoclonal antibodies (MAbs), B1.1 and COL-4, which recognize members of the CEA family were shown to react with the M_r 110,000 antigen. However, subsequent Northern blot analyses using with specific or broadly reactive CEA and NCA complementary DNA (cDNA) probes did not identify any transcripts in poly(A)$^+$-selected mRNA isolated from the human gastric cells which expressed the M_r 110,000 antigen. Only the polymerase chain reaction (PCR) method was successful in detecting an appropriately sized product for the M_r 110,000 antigen in human gastric carcinoma cells. Other physicochemical properties also suggested that the M_r 110,000 antigen may be a distant relative of the CEA gene family.

In concomitant studies, it was revealed that the level of expression of the M_r 110,000 antigen on the surface of human gastric carcinoma cells could be substantially increased with interferon-γ (IFN-γ) treatment. IFN-γ regulation 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 had been well-characterized and were expressed by human gastric carcinomas [one exception - the D612-reactive M_r 48,000 antigen]. IFN-γ administration substantially increased the expression of the M_r 110,000 antigen in six different gastric tumor cells, and de novo induced its expression in another gastric tumor cell line. Constitutive CEA and NCA was measured on the surface of five of the eight gastric carcinoma cell lines and IFN-γ treatment caused only a modest increase in their level of expression. Four other human tumor-associated antigens, TAG-72, CA19-9, D612, and 17-1A, were found either to be not constitutively expressed and/or their constitutive level of expression not enhanced as a result of IFN-γ treatment. Therefore, IFN-γ selectively upregulates the M_r 110,000 antigen and, to a lesser extent, the antigens of the CEA gene family.

The M_r 110,000 antigen which was initially described in human gastric carcinoma cells by its cross-reactivity with CEA MAbs, as well as the ability of IFN-γ to increase its level of expression was molecularly cloned and the resulting complementary DNA sequence characterized. The cDNA sequence was consistent with a 1.5-kilobase message which encoded for a 407-amino-acid polypeptide whose structural analysis was indicative of an integral membrane glycoprotein. The extracellular domain was rich in serine and threonine residues at which carbohydrate substitution is likely through *O*- and *N*-linked glycosylation. This would explain the higher molecular weight of the antigen whose polypeptide backbone is approximately 42 kilodaltons. Further computer-aided sequence analyses revealed no significant homology with any member of the CEA gene family. Cross-reactivity with MAbs B1.1 and COL-4, two anti-CEA MAbs, may be explained by the presence of CEA and normal cross-reacting antigen (NCA) homologous sites 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 in human cancer and/or as an IFN-γ-inducible gene product warrants further investigation.

From our earlier studies it was apparent that several signal transduction pathways mediated by different types of differentiation-modulating agents could enhance the expression of major histocompatibility as well as non-major histocompatibility (ie., tumor-associated) antigens on the surface of human carcinoma cells. Subsequent studies for this laboratory showed that analogues of cyclic AMP could increase CEA expression in a human colon carcinoma cell line previously shown to be interferon-resistant. Thus, signal transduction via a protein kinase pathway seemed to mediate the increases in CEA expression. A survey of different interleukins revealed that only IL-6 could increase CEA and HLA class I expression on the surface of established human colon tumor cell lines. The IL-6-mediated increase of CEA expression on the surface of a moderately differentiated colon carcinoma cell line was time and dose dependent and accompanied by increased levels of CEA-related transcripts. Those findings also formed the rationale for additional studies designed to determine whether the different types of differentiation-modulating agents could act synergistically to enhance surface antigen expression. Two obvious candidates were IFN-γ and

IL-6 since both cytokines when administered as single agents significantly increased CEA and HLA class I antigen expression. Studies were carried out to determine whether by combining those cytokines a synergistic enhancement of CEA and HLA expression could be achieved. The findings revealed that the administration of 20 units IFN-γ along with 1.7 ng IL-6, concentrations of each cytokine that individually induced minimal antigenic changes, together synergistically increased CEA and HLA class I as well as induced qualitative changes in HLA expression on WiDr human colon carcinoma cells. The magnitude of the synergistic increases in CEA and HLA class I expression were reminiscent of the level of antigen augmentation observed when administering 20- to 100-fold higher amounts of each cytokine as a single agent. Also, the addition of IL-6 potentiated the IFN-γ induction of HLA class II expression. The combined administration of IL-6 and IFN-γdid not have any additive or synergistic effects on the growth suppression of those tumor cells. Interestingly, utilization of specific neutralizing antibodies for type I interferons abrogated the increases of CEA and HLA expression seen with IL-6 treatment alone or in combination with IFN-γ. Moreover, reverse transcriptase/polymerase chain reaction analyses revealed a constitutive expression as well as a temporal increase of IFN-γ mRNA transcripts in colon tumor cells treated with IL-6. Therefore, IFN-γ production seems to play a critical role in the ability of IL-6 to upregulate antigen expression alone or in combination with IFN-γ. The findings provide insight into cytokine combinations that synergistically upregulate tumor-associated and normal HLA antigen expression on the surface of human tumor cells. Those results provide the rationale for the combined use of such cytokines to heighten tumor cell recognition in MAb- or cell-mediated-based immunotherapeutic approaches.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 09023-08 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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	Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Experimental Oncology Section

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

TOTAL STAFF YEARS: 9.4	PROFESSIONAL: 4.1	OTHER: 5.3

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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.)

Several murine monoclonal antibodies (MAbs) with selective reactivity to carcinoma associated antigens have been developed in this laboratory. These include MAbs against carcinoembryonic antigen (CEA), tumor associated glycoprotein (TAG)-72 and a 48 kD antigen expressed on the surface of normal and malignant gastrointestinal epithelium. While some of these MAbs have shown excellent tumor targeting in ongoing clinical trials, their use for in vivo diagnosis and therapy is limited because of their immunogenicity in patients. This project is aimed toward developing novel immunological reagents for the diagnosis and therapy of human cancers, using genetic engineering techniques. To reduce the potential problem of the immunogenicity of the anti-carcinoma antibodies, we earlier developed mouse-human chimeric (c) MAbs, including cB72.3, cCC49, cCC83 and cD612. To further reduce their immunogenicity in patients, one of our goals is to "humanize" MAb CC49. To that end, we wish to develop a well refined, high resolution, three dimensional structure of the antigen binding region of the antibody. In collaboration with Dr. Eduardo Padlan at NIH, we have carried out crystallographic studies and developed a primary structure of the anti-tumor MAb CC49 Fab'. In an effort to optimize the pharmacokinetics of plasma clearance, we developed novel chimeric immunoglobulin variants. We earlier expressed, in E. coli, a single chain antigen binding protein, sFv, derived from MAb CC49. Using a vector which carries the tac promoter and omp A signal, we have examined the effects of four variables on expression and accumulation of soluble CC49 sFv. Using retroviral expression constructs of the murine heavy and light chain genes of the anti-human colon carcinoma MAb D612, we engineered human colon tumor cells that constitutively expressed the D612-reactive antigen for the secretion of the D612 MAb. The antibody produced by these cells elicited ADCC mediated by NK cells against themselves as well as against the unmodified parental cells.

In our ongoing studies on recombinant baculovirus carcinoembryonic antigen, we have carried out serological and biochemical characterization of the recombinant protein. These studies demonstrated a virtual immunologic identity between the native and the recombinant molecules. The baculovirus recombinant CEA appeared to be a more potent humoral immunogen in mice than native CEA.

Major Findings :

Crystallographic Studies of MAb CC49 Fab'. In view of the potential diagnostic and therapeutic value of MAb CC49 in the management of cancer, it is important to "humanize" this antibody to render it less immunogenic while preserving its antigen-binding properties. To develop a three-dimensional structure of the antigen-binding site of MAb CC49, we have carried out crystallographic study of CC49 Fab'. A highly purified Fab' was generated from MAb CC49 and it was crystallized. Precession photographs showed that crystals possess the symmetry of the monoclinic space group $P2_1$. The size of the unit was found compatible with four Fab' molecules in the asymmetric unit. The Fab molecules are related by two approximately perpendicular pseudo-2-fold axes.

Production of the Soluble CC49 sFv in E. coli. A single-chain Fv of CC49 may have utility in diagnostic imagining of carcinomas. We, therefore, earlier constructed and expressed CC49 sFv in *E. coli*. While high levels of periplasmic expression was achieved, but the correctly processed sFv was insoluble and had to be denatured and refolded. We have, now, examined the effects of four variables on expression and accumulation of soluble CC49 sFv: (i) linker sequence joining V_L and V_H, (ii) IPTG concentration for induction, (iii) temperature, (iv) addition of sugar to the medium. We have been able to demonstrate that the yield of soluble sFv improves by the addition of 0.4M sucrose to the medium and by inducing expression with a very low concentration of IPTG (0.02 mM-0.03 mM). The increased expression of sFv was demonstrated by SDS-PAGE analysis of the periplasmic extract as well as by its competition with MAb CC49 for binding to the immobilized tumor extracts

Expression of Self-Reactive Antibody by Human Carcinoma Cells. MAb D612 reacts with a 46 kD membrane antigen expressed on the majority of human colon carcinoma cells and it inhibits growth of human colon carcinoma xenografts in mice. The D612 MAb mediates ADCC with both human lymphocytes and macrophages. To develop a new immunotherapeutic approach for the treatment of cancer based on MAb gene therapy, we wanted to study whether human colon carcinoma cells become sensitive to immune destruction through coexpresssion of both the MAb and its reactive antigen. Retroviral expression constructs of the heavy and light chain genes of MAb D612 were generated and introduced into PA317 packaging cells. Transduction of human colon carcinoma cell line LS-174T was carried out by cocultivation with packaging cells producing infectious virions carrying MAb D612 light and heavy chain genes. Immunocytochemical staining and flow cytometric analysis of transduced tumor cells revealed that > 95% of cells were positive for Ig expression and revealed murine Ig on their surfaces. Transduced LS-174T cells were found to be more sensitive to cytotoxity mediated by natural killer cells. Also the non transduced tumor cells, when cocultivated with the transduced tumor cells, became target of the ADCC mediated by human natural killer cells.

Characterization of Recombinant Baculovirus-CEA. Human CEA, a 180 kD glycosylated protein which is expressed in a wide range of human carcinomas, has been shown to be a target for active immunotherapy of human carcinomas. Human CEA could be potentially used as an immunogen in immunotherapy protocols. However, commercially available CEA may contain non specific cross-reactive antigen (NCA) and other CEA related antigens. In our ongoing studies on CEA, we recently reported the construction and purification of a recombinant source of human CEA, BVCEA-140, and NCA, BVNCA, using baculovarius expression system. We have compared a panel of 24 anti-CEA anti-NCA MAbs for their ability to bind to their recombinant CEA and NCA proteins as well as with a new 60 kD subgenomic form designated BVCEA-60. The epitope mapping studies indicate that all the CEA specific MAbs can recognize BVCEA-140. We also compared the sugar composition of BVCEA-140 to native CEA, using a lectin-linked immunoradiometric assay. The results demonstrated that both the native and recombinant baculovirus CEA contain simple high mannose carbohydrates as well as biantennary and biantennary hybrid complexes. However native CEA also contains triantennary and tetraantennary complex sugars, while the recombinant CEA molecule does not. Immunogenicity of the recombinant CEA molecules was demonstrated in mice. ELISA and Western blot analyses

were used to determine the cross-reactivity of the anti-CEA sera. Mice immunized with BVCEA-140 elicit antibodies that are reactive to native CEA. These studies thus demonstrate that although minor sugar differences do exist between native and baculovirus-derived CEA, epitope mapping demonstrate virtual immunologic identity between these two molecules, and BVCEA-140 appears to be a more potent humoral antigen in mice than native CEA.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 09025-07 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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	John Schmitz	IRTA	LTIB, DCBDC, NCI
	Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Experimental Oncology Section

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

TOTAL STAFF YEARS: 4.2	PROFESSIONAL: 4.2	OTHER: 0

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

A

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

We have investigated a potential novel approach of combining tumor -infiltrating lymphocytes and antitumor MAbs in the creation of a T cell capable of secreting antitumor Ig, in essence, creating an antitumor Ig "factory" at the tumor site. The cDNA expression construct of the chimeric D612 heavy chain and light chain gene in retroviral vectors were introduced into MOLT-4 cells and used as a model in this studies. Chimeric D612 can be expressed and secreted by human T cell line MOLT-4. The secreted Ig retained its antigen-binding properties. We have also investigated whether human T lymphocytes are able to distinguish the determinants created by point-mutated p21ras proteins from the normal ras protein. Cellular immunity to 4 synthetic peptides representing amino acids 5-17 of mutated p21 ras proteins with an exchange of normal glycine (G12) at position 12 by valine (V12), cysteine (C12) or aspartic acid (D12)was studies. Human T-cell lines from different individuals have been established by in vitro stimulation with 13-mer peptides refecting the position 12 ras point mutations. The peptide specific T cell lines were all CD3+, CD4+ and CD8- phenotypes. Induction of proliferation and secretion of IFN-γ ,IL-2 and IL-6 but not IL4 secretion were observed when the corresponding peptide was used for stimulation. Specific cytotoxic T cell activity was detected when the corresponding mutated p21ras peptide was used to pulse the target cells, or when the target cells used were transfected with the vector carrying the corresponding point-mutated p21ras protein. We have investigated the introduction and expression of human IL-6 gene in human colorectal carcinoma cells. cDNA encoding the human IL-6 gene inserted into retroviral expression vector, was introduced into HT-29 cell human colorectal carcinoma cells by lipofection. IL-6 secreted by the transfected HT-29 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 in the transfected HT-29 cells. These results suggest another potential role for the use of IL-6 gene transfer in the immunotherapy of human cancers. We studied the effects of recombinant human M-CSF (rhM-CSF) on the ADCC activity of human monocytes. These results demonstrated that rhM-CSF significantly augment ADCC activity of human peripheral blood monocytes using MAbs to human colorectal carcinoma, suggesting a potential role for rhM-CSF in cancer immunotherapy. We have also demonstrated that human TILS can be transduced with 3'end truncated IL-2 gene and produced high amounts of IL-2 in the supernatants. These results indicate the feasibility of employing adoptive immunotherapy procotols using IL-2 transduced TILS in cancer patients. T-cell lines specific to 9-mer or 10 mer CEA peptides were established from patients with carcinoma. Further analysis of these cell lines are in progress.

Major Findings

We have investigated the effects of the introduction of hrIL-6 gene on the expression of certain antigens on a huamn colorectal carcinoma cell line, HT-29. Human rIL-6 gene inserted in a retroviral expression vector,pLNCXII, was introduced into HT-29 cells by lipofection G418 secected cells were cloned and screened for IL-6 production using ELISA. IL-6 secreting clones were further analyzed by functional assay using B9 cell bioassay and a clone designated HT-29pIL-6 was establish and producing high level of IL-6 (960 pg/ml/10^6cells/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 IL-6 gene in HT-29 cells could alter the expression of cell surface antigens. The level of CEA, HLA class 1 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, an human Ig k chain. The results suggest that the change in the expression of CEA in HT-29 cells upon introduction of IL-6 gene is selective. The percent of HT-29 cells expressing HLA class 1 and HLA class II antigens remains unaltered by the introduction of IL-6 gene into the cells. The expression of ICAM-1 wasa shown to decrease slightly. The data show that the HT-29pIL-6 cell line either cloned or uncloned, expressed a higher percentage of CEA as measured by COL-1 MAb reactivity (62.1%),than either untreated (22.0%) or exogenously treated HT-29 cells (25.5%). We have investigated whether the introduction of IL-6 gene into HT-29 cells affects the expression of IL-6 receptor on the surface of HT-29 cells. Using MAb PM-1 in flow cytometric analysis to detect IL-6 receptor, we analyzed HT-29 cells and the cloned and uncloned population of IL-6 gene transfected HT-29 cells. The results show that a small percent (2.5%) of the HT-29 cells are positive for the IL06 receptor. No significant difference was observed in the perecnt of IL-6R positive cells in the IL-6 gene transsfected bulk culture (2.1%) or cloned population (2.9%). These results together with our previous report that hrIL-6 treatment of effector cells can augment ADSCC activity, provide a rationale for the use of hrIL-6 gene transfer into tumor cells as a possible role of cancer immunotherapy.

We have investigated the possibility of creating a T cell capable of screting antitumor Ig by introducing MAb D612 heavy chain (HC) and light chain (LC) gens sequentially into MOLT-4 cells. cDNA expression construct of the human-mouse chimeric D612 HC and LC genes in retrovial expression vectors pLHCXII and pLNCXII respectively, were used in this investigation. The cDNA expression construct of chimeric D612 HC, pLHCXIID612HuG1 was first introduced into MOLT-4 cells by electrophoration. MOLT-4 cells were selected in medium containing hygromycin at a concentration of 500µg/ml. Chimeric D612 LC gene, pLNCXIID612Huk was transduced into the D612 HC-transfected MOLT-4 cells by cocultivation with PA317pLNCXIID612Huk cells, an amphotropic retrovirus packaging cell line productively transfected with the retroviral expression vector pLNCXIID612Huk. MOLT-4 tranductants were selected in selective medium containing G418 at an active concentration of 1 mg/ml. Supernatant from the chimeric D612 HC and LC genes transfected cultures were assayed for D612 reactivity and those positive for D612 reactivity were designated as MTcD612. A clone designated clone 6 was found to secrete the highest level of chimeric D612 (0.25 µg/ml per 10^6 cells in 72 h). 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 sililar for both the MTcD612 and the parental MOLT-4 cells. Ig secreted by MTcD612 was purified and separated by 3-12% 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. Completion inhibition assay shown that chimeric D612 secreted by MTcD612 cells was able to completely block the binding of the ^{125}I-labeled parental D612 MAb. The specificity of the chimeric D612 MAb was further analyzed by ELISA with the use of antigen extracts from various human tumor cell lines. The results suggested that the reactivity of the chimeric MAb was similar to the parental D612 MAb. Positive binding was observed with tumor cell extracts from LS174T and GEO but not with tumor cell extracts obtained from HT-29, WiDr, A375, and MCF. Studies were conducted to determined whether the chimeric D612 scdreted by MOLT-4 cells could mediate ADCC activity against human tumor cells. The results showed that the lytic activity mediated by chimeric D612 was at least as efficient and possibly higher 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 anticancer antibodies 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 for 72 h with rhM-CSF at various concentration and then used as effect cells in a 72 h ^{111}In release assay. cell lines LS-174T, CBS(human colorectal carcinomas) and KLE(poorly differentiated human endometrial carcinoma) were used as targets to react with anti-colorectal carcinoma MAbs D612, native CC49 and chimeric CC49. Initial experiments using 1 µg/well of D612 MAb to determine whether the ADCC mediated by monocytes could be augmented by rhM-CSF using LS174T as target. Effectors were incubated with rhM-CSF at concentrations of 50, 100, 200, 400 U/ml for 72 hr prior to ADCC assay. Optimal activity was observed at concentration of 100 U/ml of rhM-CSF and therefore this concentration was chosen for the remaining experiments. Treatment of effector cells with 100 U/ml of rhM-CSF for 72 hr did not increase the number of effector cells in the assay. Treatment of effector cells with rhM-CSF for 72 hr did not increase the number of effector cells in the assay. In the absence of rhM-CSF, monocytes lysed 41% and 22% of LS174T target in the presence of D612 MAb at effector : target ratios of 50:1 and 25:1 respectively. The corresponding lysis in the presence of UPC-10 control MAb was 16% and 5% for the 50:1 and 25:1 effector : target ratio respectively. Upon incubation with rhM-CSF, increase in ADCC was observed at 100 U/ml to 400 U/ml. No increase in nonspecific cytotoxicity following exposure of monocytes was observed. Effector cells were incubated with 100 U/ml of rhM-CSF for different times and tested against LS174T target cells in the presence of 1 µg/well of D612 MAb. We found that rhM-CSF had enhanced ADCC by 24 hr of incubation of effectors and was maximal at 5 days of incubation (from 40% to 83%). A significant augmenation in ADCC activity could be otained by treatment of monocytes with rhM-CSF for 3 days and therefore this was chosen as the pretreatment time for the remaining experiments. Monocytes from seven donors were tested for their ability to respond in ADCC after pretreatment with rhM-CSF. ADCC activity was enhanced by pretreatment of rhM-CSF in all seven donors. However, the degree of augmentation varied among different donors as did the base line ADCC activity in the absence of rhM-CSF. Augmentation of nonspecific cytotoxocity was not observed with preincubation of monocytes with rhM-CSF. The ability of rhM-CSF to augment the ADCC activity using various target cells was investigated. Two additional target cell lines (CBS and KLE) and two other MAbs to colorectal carcinomas (native CC49 and chimeric CC49) were used in the ADCC assay. A375 cell line was used as control. It was demonstrated that rhM-CSF could augment ADCC activity when CBS cells but not A375 cells were used as target. Cytotoxicity was augmented from 45% to 82% (E:T = 50:1), 31% to 57% (E:T = 25:1), 21% to 39% (E:T = 12.5:1) and 11% to 31% (E:T = 6.5:1). No rhM-CSF related augmentation was observed when A375 was used as target. rhM-CSF could also increase ADCC activity with both MAbs CC49 and chimeric CC49 using KLE as target cells. The effect of rhM-CSF on the concnentration of D612 MAb required to mediate ADCC activity was investigated. D612 MAb at various concentrations (1 µg, 0.1 µg, 0.01 µg, 0.005 µg and 0.001 µg/well) were used in the ADCC assay. Precubation of effector cells with rhM-CSF could decrease the amount of D612 MAb required to give the equivalent level of ADCC activity. For example: using rhM-CSF treated effectors, D612 MAb at a concentration of 0.005 µg/well could mediate a level of ADCC activity similar to that seen using 0.1 µg/well of D612 MAb and effector cells not preincubated with rhM-CSF. In order to ascertain that the increase in ADCC activity after pretreatment of effector cells with rhM-CSF was caused by the additional of rhM-CSF to the culture medium, rhM-CSF neutalization antibody was added to the medium during the pretreatment time. The addition of anti-rhM-CSF antibody completely inhibited the rhM-CSF mediated augmentation of ADCC activity. The inhibition was dependent on the dose of anti-rhM-CSF antibody. The effect of rhM-CSF on ADCC activity was inhibited to control level (from 72% to 41%) when 150 µg of anti-rhM-CSF antibody was used. ADCC decreased from 72% to 51% when 75 µg of anti-rhM-CSF antibody was used for treatment of effector cells. The effect of pretreatment of monocytes with rhM-CSF on the secretion of TNF-a and IL-6 was investigated. The levels of TNF-a and IL-6 secreted by rhM-CSF treated monocytes were significantly higher compared to the untreated monocytes. Furthermore, significant levels of TNF-a and IL-6 were also detected in a 24 hr culture supernatant of the washed rhM-CSF treated monocytes.

To investigate whether rhIL-4 could augment the induction of ADCC activity mediate by monocytes preincubated with rhM-CSF, human peripheral blood monocytes were costimulated with 0, 0.1, 1.0 and 10 ng/ml of rhIL-4 plus 0 or 100 U/ml of rhM-CSF for 72 hr prior to ADCC assay. The ADCC obtained from two donors. Preincubation of monocytes with 0.1, 1.0, and 10 ng/ml of rhIL-4 plus 100 U/ml of rhM-

CSF significantly augmented the ADCC activity as compared to rhM-CSF pretreatment alone. Pretreatment with rhIL-4 alone at 0.1, 1.0 or 10 ng/ml did not increase the ADCC activity in either donor. To ascertain whether rhIL-4 could augment the induction of ADCC activity mediated by monocytes exposed to suboptimal doses of rhM-CSF, human monocytes were costimulated with 0 or 10 ng/ml of rhIL-4 plus 0, 25, 50 or 100 U/ml of rhM-CSF for 72 hr prior to ADCC assay. Significant enhancement of ADCC was observed when monocytes were preincubated with 50 U/ml of rhM-CSF plus 10 ng/ml of rhIL-4. No augmentation of monocytes ADCC was detected when 50 U/ml of rhM-CSF was used alone. This results demonstrated that rhM-CSF significantly augment ADCC of human peripheral blood monocytes using MAbs to human colorectal carcinoma suggesting a potential role for rhM-CSF in cancer immunotherapy.

Point mutated ras oncogenes are found in a wide range of human tumors, including pancreatic, colorectal, lung and thyroid carcinomas as well as melanomas and leukemias. The vast majority of mutations in the ras p21 protein are found at position 12, with glycine (G) changing to valine (V), cystein (C), or aspartic acid (D) constituting most of the mutations. Previous studies have shown that both murine and human T-cell lines can established upon stimulation with peptides reflecting the ras mutations at position 12, 13 and 61. We have attempted to further define the specificity of human T-cell lines to various ras peptides; to determine the cytokine production profile of established T-cell lines; to determine if any cytotoxic activity of T- cell lines could be defined, either by pulsing autologous B-cells with peptides reflecting point mutated ras, or by transducing the corresponding point mutated ras gene into autologous B-cells using retroviral vectors. Four human T-cell lines from different individuals have been established by in vitro stimulation with 13-mer peptides reflecting the position 12 ras point mutations. All T-cell lines were of the CD3+, CD4+ and CD8- phenotype. Specificity of the T-cell lines to stimulating peptide was observed as a proliferative response using the corresponding peptide but not with peptides reflecting other mutations or normal ras (G12). Human T-cell lines (specific for V12, C12 or D12 mutations) all respond to corresponding peptides with the production of IFN-g and IL-6, but not IL-4. Using V12 specific T-cell lines, it was demonstrated that EBV-transformed autologous B-cell lines could be lysed when incubated with the V12 peptide and not other p21 ras-derived synthetic peptides. Moreover, when the autologous EBV-transformed B-cell line was transduced with p21 ras oncogene containing the V12 mutation in retroviral vector, these cells became susceptible to lysis by the autologous V12 peptide specific T-cell line. These stidues indicate that the point mutated ras gene product may be processed in an appropriate manner as to be a target for T-cell mediated cytotoxicity. The point mutated ras oncogene product may thus potentially serve as a target for T-cell mediated cytotoxicity for a range of human cancers using immunotherapy protocols.

We have also investigated if tumor-infiltrating lymphocytes (TILS) can be genetically modified to produce IL-2. We have modified melanoma TILS with IL-2 gene and clarified functional characteristics of the TIL transductants. TILS transduced with 3' end-truncated IL-2 gene (480bp) produced high amounts of IL-2 detected in supernatants when compared to TILS transduced with the native IL-2 gene containing 3' end-AT-rich sequences (650bp). The level of IL-2 in supernatants was augmented by the addition of anti-Tac antibldy to block the consumption of IL-2 by the TILS. These TILS could proliferate autonomously in the absence of exogenous IL-2, and the proliferation of TILS could be completely blocked by anti-IL-2 antibldy or anti-IL-2 receptor antibody. Thus TILS transduced with IL-2 gene can proliferate through the autocrine loop. The expression of IL-2 from TILS transduced with the IL-2 gene was downregulated after 2-3 weeks of G418 selection. These studies indicate the feasibility of transduction and expression of truncated 480bp IL-2 gene into TILS and the possibility of employing adoptive imunotherapy protocol using TILS modified with IL-2 gene.

We have also investigated the primary response to CEA peptides of PBL derived from normal individuals and cancer patients. T-cell lines to CEA peptides were established from patients with carcinoma using 9-mer or 10-mer CEA peptides. These T-cell lines were peptide specific as assayed by cytokine release and cytotoxicity assays. Further characterization of these cell lines are in progress.

Publications

Tsang KY, Kashmiri SVS, Qi CF, Nieroda CA, Calvo B, DeFilipp 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. Immunology Letters 1993; 36:179-86.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 09028-04 LTIB

PERIOD COVERED: October 1, 1993 to September 30, 1994

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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)

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Continued on page 2

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Experimental Oncology Section

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

TOTAL STAFF YEARS: 8.4	PROFESSIONAL: 6.4	OTHER: 2.0

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

A

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

Certain tumor associated antigens(TAAs) represent potential targets for specific active immunotherapy. Our laboratory has been evaluating the safety, toxicity and immunogenicity of using recombinant vaccinia viruses, expressing human tumor associated antigens, as immunogens for the treatment of human carcinomas. We have constructed, characterized and determined the safety and immunogenicity of two recombinant vaccinia viruses in murine tumor models as well as rhesus monkeys. The first recombinant vaccinia virus expressed human carcinoembryonic antigen(CEA) and the second vaccinia virus expressed human prostate specific antigen(PSA). CEA is a 180Kd glycoprotein which is overexpressed in human colorectal, gastric, pancreatic, breast and non small cell lung carcinoma. PSA is a glycoprotein of 30-33 Kd which is expressed in prostrate carcinoma. It is unclear whether these TAAs are immunogenic in humans. Humoral and cell mediated responses to these tumor antigens have not been well documented in normal or cancer patients. The presentation of these TAAs in the context of Class I MHC might increase their immunogenicity and represent a logical approach to inducing anti CEA and PSA responses for tumor immunotherapy. We have recently shown that CEA could be used as a target for active immunotherapy. Anti-tumor activity was demonstrated in an animal tumor model by immunization of mice with the recombinant vaccinia virus expressing human CEA. This recombinant vaccine was also shown to induce both cell-mediated and humoral CEA specific immune responses in mice and nonhuman primates. The recombinant CEA vaccine has been evaluated in a Phase I clinical trial at the NCI from May to December of 1993. Twenty six patients who had CEA expressing carcinomas entered the trial . Seven patients received Dose level I (2X105 PFU), seven patients received dose level II (2X106 PFU) and 12 patients received dose level III (1X107 PFU). These doses were given three times by skin scarification four weeks apart. These studies have shown that the recombinat CEA vaccinia virus can be safely administered to cancer patients with no toxicity and that this vaccine can serve as an immunogen for repeated exposures to a specific tumor associated antigen. Clinical and immunological evaluations for the presence of CEA specific humoral and cell mediated responses are being performed. A recombinant PSA vaccinia virus has been constructed and evaluated for its safety and immunogenicity in a murine tumor model expressing human PSA and in male rhesus monkeys. This vaccine was shown to have no toxicity in these animal models. Monkey toxicity studies have shown that blood counts, differentials and hepatic and renal chemistries remained normal throughout the study. These animals are being evaluated for PSA specific cell mediated responses.

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Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

MAJOR FINDINGS

Phase I Clinical Trials

We have shown that CEA can be a target for active specific immunotherapy. Anti-tumor activity in an animal tumor model was induced by immunization of C57BL/6 mice with a recombinant vaccinia virus expressing human CEA. This vaccine was shown to induce both humoral and cell-mediated immune responses specific for CEA in mice and nonhuman primates. This recombinant CEA vaccinia virus has now been evaluated in Phase I clinical trials on patients with CEA expressing adenocarcinomas. In collaboration with Dr. M. Hamilton, COP, DCT, NCI, twenty six patients entered a phase I clinical trial from May to December of 1993. Three dose levels of the recombinant vaccine were evaluated for safety, toxicity and CEA specific immune responses in these patients. Seven patients received dose level I which was $2X10^5$ PFU (plaque forming units) , seven patients recived dose level II, $2X10^6$ PFU and 12 patients received $1X10^7$ PFU. These immunizations were given by dermal scarification three times four weeks apart.. The patients ranged in age from 32-77 years old with a median age of 54. There were 12 females and 14 males enrolled in this study. 17 patients had colon cancer, 3 had lung cancer, 2 breast cancer and 4 patients had other tupes of CEA expressing carcinomas. All patients had prior smallpox immunizations. After the first treatment all patients had a reaction with a 1-2 cm bulla or pustule and local erythema. Some patients had symptoms of fever, fatigue and malaise. Approximately 75% of the patients receiving a second and 24% of patients receiving a third immunization also had a positive reaction. Side effects were limited to the pustular response at the skin site. Hematologic, renal and liver function toxicities were < grade 1. 13/14 patients on the first two dose levels had progressive disease and one had stable disease. It is too early to evaluate the patients at the third dose level . Anti vaccinia and anti CEA T cell and B cell activity are being monitored in these patients. Recombinant vaccinia virus expressing CEA can be safely administered and can serve as an immunogen for repeated exposure to a tumor associated antigen gene product. Clinical and immunological assessment of this recombinant CEA vaccinia virus is proceeding in these patients.

Serological and Biochemical Characterization of Recombinant Baculovirus CEA

The evaluation of CEA specific humoral and cell mediated immune responses in mice, monkeys and humans after rV-CEA immunizations relies on large quantities of purified CEA. We have recently demonstrated that the commercially available sources of CEA which is purifed from liver metastasis and supernatants of tissue culture fluid may contain NCA and other CEA-related antigens. We recently reported the construction and purification of a recombinant source of human CEA and NCA using a baculovirus expression system. The baculovirus protein expression system has been used to express glycosylated proteins. A number of studies have demonstrated that the recombinant proteins produced by the baculovirus may be immunologically indistinguishable from the native molecule. Our study was undertaken to investigate if the

recombinant source of CEA retained the same biochemical and antigenic properties as native CEA. This recombinant molecule could potentially be useful as a defined laboratory reagent for immunological assay systems as well as an immunogen in immunotherapy protocols.

We have compared a panel of 24 anti-CEA and anti-NCA monoclonal antibodies for their ability to bind these recombinant CEA and NCA proteins. The epitope mapping studies indicate that all the CEA specific MAbs can recognize the recombinant protein, BV140. We also compared the sugar composition of BV-140 to native CEA, using lectin-linked immunoradiometric assays. The results demonstrated that both native and recombinant baculovirus CEA contain simple high mannose carbohydrates. They also contain biantennary and biantennary hybrid complexes. However, native CEA also contains triantennary and tetraantennary complex sugars while the recombinant CEA does not. Immunogenicity of the recombinant CEA molecules was demonstrated in mice. ELISA and western blot analyses were used to determine the cross reactivity of anti-CEA sera. Mice immunized with BV-140 elicit antibodies that are reactive to native CEA. These studies also demonstrated that although minor sugar differences do exist between native and baculovirus derived CEA, epitope mapping with a panel of 24 anti-CEA MAbs (recognizing at least 10 independent CEA epitopes) demonstrated virtual immunologic identity between these two molecules. Moreover, BV-140 appears to be a more potent humoral immunogen in mice than native CEA. Thus, these recombinant molecules can serve as a reproducible purified homogenous source for laboratory reagents to serve as standards in CEA serum assays and *in vitro* analysis of cell mediated immune responses to CEA after active specific immunotherapy. Studies are in progress to evaluate the ability of BV-140 to be used as an immunogen alone or as a "boost" after a primary immunization with rV-CEA in an experimental CEA expressing tumor model.

Construction and Characterization of a Recombinant Vaccinia Virus Expressing Human Prostate Specific Antigen (PSA)

A 1.5 kb cDNA clone containing the complete coding sequence of PSA was obtained by r-PCR using total RNA from the human prostate cell line, LNCAP and PSA specific primers. The cDNA was inserted into the vaccinia virus transfer vector T116. The expression of the PSA gene is under the control of the vaccinia virus early promoter P40. A recombinant vaccinia virus expressing PSA was obtained by homologous reombination of the plasmid with Wyeth vaccinia virus. Recombinant vaccinia virus were selected by expression of Lac Z using a blue plaque assay. Recombinant plaques were purified through three rounds of plaque purification and analysed for the expression of PSA by western blot analysis. Supernatant fluid as well as well as cell extracts from BSC-1 cells infected with rV-PSA showed an immunoreative band at 30-32Kd using either a polyclonal rabbit antisera against human PSA or a mouse monoclonal antibody specific to human PSA. The expected genomic structure of the recombinant vaccinia virus was confirmed by restriction endonulcease digestion and Southern blot analysis using PSA as a probe. The rV-PSA contained no genomic rearrangements. The recombinant PSA vaccinia virus was developed , amplified and GMP grade vaccine made at Therion Corporation, Boston MA. This vaccine was used as the source of immunogen in all of our murine and nonhuman primate studies. Rodent tumors do not express PSA. In order to test the efficacy of a PSA recombinant vaccinia virus a rodent tumor model expressing PSA was developed. The MC-38 mouse colon adenocarcinoma cell line was transduced with a retroviral vector containing human PSA. Recombinant colonies were selected in G418, cloned and tested for their expression of PSA. Several clones which expressed high levels of PSA were extensively characterized . These clones formed tumors when transplanted by subcutaneous injection into C57BL/6 mice. These PSA+ tumors grew at a somewhat slower rate than the MC-38 retriviral transduced control cell line. However , the tumors from both PSA transduced cells and those from retrovirally transduced control cells eventually killed the mice. These cell lines will be used as targets to evaluate the anti-tumor effect of the rV-PSA vaccine. This vaccine will also be evaluated for its ability to elicit humoral and cell mediated responses in C57BL/6 mice.

Safety, toxicity and immunogenicity of rV-PSA was evaluated in 12 male rhesus monkeys. The prostate gland of the male rhesus monkey expresses a protein that is immunologically related to human PSA. Southern blot hybridization to rhesus monkey DNA using human PSA as a probe and subsequent nucleic acid sequence analysis of the PSA gene has demonstrated 80-90% sequence identity between rhesus monkey and human PSA. Three groups of four animals were given various dose levels of either Wyeth or rV-PSA by dermal scarification. One animal out of each group was prostecomized. $1X\ 10^8$ PFU of Wyeth vaccinia virus was administered three times at 4 week intervals to Group I. Group II received $1X10^7$ PFU of rV-PSA three times at four week intervals. Group III received $1X10^8$ PFU of rV-PSA using the same immunization schedule. Monkeys receiving V-Wyeth were compared to monkeys immunized with rV-PSA 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. The sera from these animals was also tested by ELISA for immunoreactivity to PSA. None of the rV-PSA immunised animals had IgG antibodies to PSA. PSA antibody levels could not be detected in the sera even after the second and third immunization. It appears that rV-PSA was unable to elicit a humoral response in these monkeys. The rV-PSA vaccine was not toxic in these animals. Blood counts, renal and hepatic chemistries remaied normal throughout the study. We are currently evaluating cellular immune responses specific to PSA in these animals.

PUBLICATIONS

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 1993;2:79-94.

Bei R, Kantor J, Kashmiri SV, Schlom J, Serological and biochemical characterization of recombinant baculovirus carcinoembryonic antigen. Mole Immunol (In press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 09029-02 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Experimental Oncology Section

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

TOTAL STAFF YEARS: 6.3	PROFESSIONAL: 4.2	OTHER: 2.1

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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.)

We have continued to focus on the growing area of experimental and clinical immunotherapy of human carcinoma. Central to its investigative design is the biological application of recombinant poxviruses, recombinant proteins and/or peptides as "tumor vaccines", which specifically target those cancers. Our studies emphasize a basic and fundamental understanding of the mechanisms of action and immunological impact of such vaccines in preclinical animal models and now in patient clinical trials. Much of this work has concentrated on carcinoembryonic antigen (CEA) and the point-mutated *ras* p21 oncogene. The over-expression of tumor-associated antigen (Ag) (i.e., CEA) and/or the neo-expression of tumor-specific epitopes (i.e., point-mutated *ras* p21) may represent selective or unique targets for immune recognition, particularly by T-lymphocytes which have been implicated as important elements for host defense against malignancy. Here, in a murine model, we explored and characterized distinct effector properties of host-derived T-lymphocytes reactive to mutated *ras* peptides. BALB/c mice (H-2d) were inoculated with a purified peptide, 13 amino acids in length, reflecting positions 5–17 of the normal (G12) or a mutated (V12) *ras* protein commonly found in human carcinomas. A T-cell line and clonal derivatives of that line were established to the *ras*(V12) peptide, which displayed an ab-TCR+, CD3+, CD4+, CD8- phenotype and expressed Ag specific proliferation. Cytokine secretion revealed TNF, GM-CSF, IFN-γ, and/or IL-2 patterns, consistent with the CD4+ Th1 subtypes. Moreover, Ag-specific cytotoxicity was demonstrable against syngeneic lad-bearing tumor cells either incubated with exogenously bound *ras*(V12) peptide or transduced with the point-mutated Ki-*ras*(V12) oncogene. In parallel experiments, we have begun to examine the feasibility for the induction of anti-ras CD8+ cytotoxic T lymphocytes (CTL). We have identified two mutant *ras*(V12) sequences, 4-12 (9-mer) and 3-12 (10-mer), as potential CD8+ epitopes for murine H-2Kd and confirmed their MHC class I binding activity by a functional competition assay. We plan to explore these peptides as well as vaccinia recombinants for their *in vivo* CD8+ immunogenicity. Finally, we have begun to explore the safety and efficacy of a vaccinia recombinant expressing human CEA in phase I clinical trials. Here, we examined its impact on cell-mediated immunity and found evidence, in about 50% of the patients, for the augmentation of pre-existing anti-vaccinia responses. The extent of any CEA-specific immunity is unclear and still under investigation.

Major Findings

The *ras* p21 proto-oncogenes encode proteins that have been implicated in the differentiation and regulation of cellular growth and function. However, alterations in *ras* p21 proteins resulting from genetic mutations have been associated with both rodent and human cancers. Such mutations have been identified in a broad spectrum of carcinomas and result in single amino acid substitutions in the native protein, notably at positions 12, 13, and 61, which alter the GTPase activity of the protein and confer transforming activity and the malignant phenotype. Thus, mutated *ras* p21 proteins may bear unique antigenic determinants for immune recognition and attack, particularly by T-lymphocytes, which are thought to play an important role in host antitumor activity . Furthermore, the *ras* proto-oncogenes are highly conserved in evolution between mice and humans, making the mouse a potentially useful model to study *ras* p21 oncogenesis and the role of host antitumor immune responses.

Here, we focused on the substitution of Gly (G) to Val (V) at position 12 in the mutated *ras* p21 protein, which is found in many human carcinomas. We developed a murine model (BALB/c, H-2^d) to evaluate the functional and phenotypic properties of the T-cell response induced by peptides reflecting mutated *ras* p21. We demonstrate: (1) antigen (Ag)-specific T-cell proliferation in mice immunized with a 13-mer *ras* (V12) peptide encompassing positions 5 to 17, without significant cross-reactivity to homologous peptides reflecting alternative position 12 mutations, including Cys12, Arg12, or Asp 12, as well as to normal *ras* (G12); (2) *ras* (V12) peptide-specific $CD4^+$ T-cells (line/clones) were established *in vitro* which produced tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-g (IFN-g), and/or interleukin-2 (IL-2); (3) such $CD4^+$ T-cells, which produced IFN-g, expressed Ag-specific cytolytic activity against syngeneic Ia^d tumor cells (i.e., "A20" B cell lymphoma) incubated with the appropriate exogenous peptide; (4) a $CD4^+$ T-cell clone, which did not produce IFN-g, lacked cytotoxic function but underwent lymphoblastic transformation and secreted cytokines such as TNF and GM-CSF in response to Ag-bearing tumor cell stimulation; and (5) $CD4^+$-mediated cytotoxicity was determined to be major histocompatibility complex (MHC) class II-restricted, as revealed by the absence of lysis against class II^- targets, the capacity to inhibit Ia^d-specific killing with anti-class II MAb, and the induction of lysis using L-cell transfectants that expressed the appropriate restriction element that mapped to $EaAb^d$.

Taken collectively, these data demonstrate that mutated *ras* peptides are immunogenic in an appropriate host and can activate a repertoire of T cell responses thought to be important in antitumor activity. The relative success of peptide-based immunotherapy is dependent upon the capacity of these peptide-induced T cells to productively recognize and respond to autologous tumor expressing endogenous *ras* Ag. To that end, we introduced the *ras* p21 oncogene encoding the corresponding point mutation by retrovirus transduction into the same tumor population (i.e., A20 cells) shown to be susceptible to lysis with exogenous peptide. Resulting transductants were screened and evaluated by northern and PCR analysis for RNA (message), flow cytometry for MHC class II expression and, lastly, for lytic sensitivity against these *ras* peptide-specific $CD4^+$ effectors. We found specific $CD4^+$-mediated lysis *in vitro* against *ras* (V12)-transduced A20 tumor cells in the absence of exogenous peptide, but not against the vector or non-transduced targets as controls. As expected, lysis of these *ras*(V12)-transduced tumor cells represented a fraction of activity (< 50% lytic efficiency) seen under maximal conditions with exogenous peptide. The control targets were efficiently killed in the presence of exogenously bound peptide, confirming their general lytic sensitivity and competent expression of functional MHC class II molecules. Thus, these observations support the hypothesis for $CD4^+$ T-cell recognition of MHC class II^+ tumor cells expressing endogenous *ras* Ag, and provide the rationale for future experiments exploring the *in vivo* efficacy of peptides and/or adoptive transfer of Ag-reactive T cells for immunoprotection and immunotherapy.

In the studies just described, we found little evidence for the stable induction of $CD8^+$ activity, which together with $CD4^+$ responses would likely promote a more effective and potent antitumor immune response. Thus, in parallel to the $CD4^+$ objectives, we began to explore possibilities for $CD8^+$ T cell activation against the same antigenic region (V12). We identified two mutated *ras* sequences, 4-12 (9-mer) and 3-12 (10-mer) as potential $CD8^+$/cytotoxic epitopes in the murine H-2^d (BALB/c) system. These predictions, in part, were based on the modeling and identification of known MHC class I binding concensus motifs for murine H-$2K^d$, which describes an 8 to 10-mer peptide with Tyr at the N-terminal position 1or 2 and a hydrophobic residue, such as Val or Leu, at the C-terminal. (No known concensus motifs for murine H-$2L^d$ or H-$2D^d$ were found around *ras* V12). Moreover, the possibility that the mutated residue would serve as the C-terminal anchor may underlie the basis for at least one mechanism for anti-*ras* $CD8^+$ immunogenicity and epitope-specific reactivity. Similarly, this rationale can be applied in the human system for *ras* peptide sequence 5-12, which would satisfy a concensus motif for HLA-A2. Thus, we propose the hypothesis that for $CD8^+$ CTL induction, the point mutation in the *ras* p21 oncogene at codon 12 introduces a new amino acid residue (i.e., V12) which now confers MHC class I binding activity in an otherwise non-binding, non-antigenic peptide that lacks a necessary anchor motif (i.e., G12). To test this hypothesis, we first established a functional MHC class I binding assay and examined the efficiency of *ras* peptide binding by functional competition of a known H-$2K^d$ viral peptide-restricted $CD8^+$ CTL response. We found that both mutated *ras* peptides 4-12 (V12) and 3-12 (V12) significantly blocked CTL lysis; whereas, the normal (G12) counterpart sequences failed to do so as well as other mutated *ras* 13-mer peptides, including 3-15, 4-16 and 5-17. These data indicate that 4-12 (V12) and/or 3-12 (V12) peptides bind to MHC class I H-$2K^d$ and, further, support the contention that they may be putative anti-*ras* $CD8^+$ CTL epitopes. In future experiments, we plan to explore this hypothesis using several approaches: (1) administration in new adjuvant systems, either singly or in combination with a *ras* $CD4^+$/helper epitope (i.e., 5-17); (2) immunization with "peptide-pulsed" autologous Ag-presenting cells; and (3) encapsulation and delivery in liposomes. In addition to peptide-based strategies, we plan to examine the capacity of *ras*-vaccinia recombinants to induce $CD8^+$ CTL responses.

A second major effort has been dedicated to the immunological evaluation of patients receiving the recombinant vaccinia virus-CEA vaccine in phase I clinical trials. Previously, we have described our preclinical observations which helped contribute to the initiation of this protocol. Twenty-six patients with metastaic adenocarcinomas of the gastrointestinal tract (primarily colon), breast or lung entered this study and were separated into three dose escalation groups: 2×10^5 (level I, 7 patients), 2×10^6 (level II, 7 patients) and 1×10^7 (level III, 12 patients) plaque-forming units (PFU). Each patient completed "3 cycles" of therapy, separated at 4 week intervals. Heparinizaed whole blood was drawn prior to the first vaccination and immediately preceding each successive vaccination. Thus, post-vaccination specimens were obtained at 4 week intervals, which was considered as an adequate time to evaluate for the induction or modulation of cell-mediated immunity (CMI) to vaccinia virus and/or CEA antigens. (A multitude of other analyses were also performed in parallel, which involved other collaborators). Here, lymphoblastic transformation *in vitro* was examined as a specific and sensitive measurement for the acquisition of CMI. Overall, at this time, we demonstrate the following: (1) mitogen responses to PHA and PWM revealed general immune competence of T and B cell function, respectively, in all patients, pre- and post-therapy; (2) 19/26 patients with pre-existing CMI to vaccinia Ag (i.e., stimulation index ≥ 3); (3) 4/7, 5/7, and 4/12 levels I, II, III patients, respectively, showed enhanced immune reactivity to vaccinia Ag after the first or second vaccination; (4) no obvious evidence for the induction of anti-CEA immunity to at least soluble CEA protein; (5) correlations between immune responsiveness and clinical course of disease is in progress. Thus, it appears that this vaccinia recombinant can be safely administered as a vaccine to humans and that in some, but not all patients, pre-existing anti-vaccinia immunity can be boosted. We plan to explore further the impact of vaccination on the generation of CEA-specific immunity *in vitro* using synthetic CEA peptides or fowlpox/avipox recombinants. In contrast to soluble protein, these latter means for Ag presentation may better mimic what the host T cells see *in vivo*.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 09003-12 LTIB

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October 1, 1993 to September 30, 1994

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)

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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: 2.0	OTHER: 2.0

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

B

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

Transforming growth factor a (TGFα), 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 TGFα and AR can bind to the EGF receptor (c-*erb* B). TGFα 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, TGFα 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 TGFα. Furthermore, overexpression of a human TGFα cDNA in these cells leads to their in vitro transformation. Addition of an anti-EGF receptor blocking antibody or an anti-TGFα neutralizing antibody can partially or completely inhibit the growth of the Ha-*ras* or TGFα transformed mammary cells sugesting 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 TGFα 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 TGFα 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 TGFα promoter contains a *cis*-acting estrogen-responsive element(s) (ERE). MCF-7 or ZR-75-1 cells were infected with a recombinant amphotropic TGFα antisense mRNA expression vector. Expression of this antisense mRNA lead to a reduction in estrogen-induced TGFα 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 80% of primary human breast tumors at a level that exceeded the level found in adjacent normal mammary epithelium.

Major Findings

To determine if endogenous TGFα might be synthesized *in vivo* in the mammary gland and to ascertain if TGFα 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 TGFα-specific antisense RNA riboprobe to detect TGFα mRNA transcripts. Quantitation of the relative levels of TGFα 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 TGFα-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 TGFα antisense riboprobe. Expression of TGFα 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 TGFα 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 TGFα 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 TGFα 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 TGFα 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 TGFα mRNA expression. As in the rat mammary gland, TGFα mRNA expression could be localized to the epithelial cells of both the ducts and lobules. The level of TGFα mRNA expression increased approximately 2-fold in the epithelial cells during midpregnancy. These results suggest that TGFα 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 TGFα in the lactating mammary gland could also account in large part for the substantial amount of immunoreactive and bioactive TGFα which we had been able to detect in human and rodent milk.

We have demonstrated that TGFα 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 3×10^5 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 TGFα mRNA expression and TGFα 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 TGFα and that TGFα 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 α (HRG α) 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 TGFα 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 TGFα cDNA to assess the transforming potential of this gene. Overexpression of the TGFα 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 TGFα in these cells. The TGFα 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 TGFα transformed MCF-10A cells transformed cells could be inhibited with either an anti-EGF receptor blocking antibody or with an anti-TGFα neutralizing antibody demonstrating that TGFα is functioning through an external autocrine loop to regulate the proliferation of these transformed cells. Collectively, these results demonstrate that both TGFα 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, TGFα, HRG α 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, β-casein. HC11 cells transformed with an activated human Ha-*ras* gene or by overexpression of the human TGFα gene are no longer able to synthesize β-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 β-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 TGFα transformed HC11 cells to respond to lactogenic hormones, suggesting that secreted TGFα is acting through an external autocrine pathway to negatively regulate β-casein expression through the EGF receptor. This is also supported by the observation that exogenous EGF or TGFα can antagonize the inductive effect of these lactogenic hormones on β-casein expression in the parental HC11 cells. In addition, the data suggest that activation of the EGF receptor by EGF or TGFα 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 since184A1N4 (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, TGFα or basic or acidic fibroblast growth factor (FGF) but not in response to either insulin, insulin-like growth factor-I (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 mammoplasty tissue contains biologically active basic FGF and the cells express specific basic and acidic FGF mRNA transcripts but not mRNA for TGFα. In contrast, the c-*myc* and SV40 T overexpressing A1N4 cells express TGFα 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 TGFα mRNA and TGFα 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 17β-estradiol (E2), whereas in ER-negative breast cancer cell lines such as MDA-MB-231 or MDA-MB-468 cells basal levels of TGFα 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 TGFα expression through the TGFα promoter/enhancer region, MCF-7 and ZR-75-1 cells were transiently transfected with plasmids containg an 1140-bp fragment of the human TGFα 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 TGFα 5′-flanking region is an ER mediated response. Using various sized fragments of the TGFα 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 TGFα, MCF-7 , ZR-75-1, MDA-MB-468 or ER-negative HS-578T cells were infected with a replication detective, recombinant amphotropic retrovirus containing a 406-bp fragment of the coding region of the human TGFα 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 TGFα antisense mRNA. Infected MCF-7 or ZR-75-1 cells expressed the TGFα antisense mRNA, exhibited a 60% to 70% reduction in E2-stimulated TGFα mRNA expression and TGFα 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 TGFα antisense mRNA but not in antisense infected ER-negative HS-578T breast cancer cells which do not express endogenous TGFα. In addition, in infected MCF-7 cells expression of the TGFα 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 2×10^4 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 1.2×10^6 EGF receptor sites/cell. These EGF receptor overexpressing clones possesed functionally normal EGF receptors since they could be autophosphorylated in response to exogenous EGF and could transphosphorylate the $p185^{erb\ B\text{-}2}$ 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.

TGFα 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 TGFα (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,TGFα and CR-1 could be detected in nearly 80% of 68 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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Normanno N, Qi, C-F. Gullick W.J., Persico G, Yarden Y, Wen D, Plowman G, Kenny N, Johnson G, Kim N, Brandt R, Martinez-Lacaci I, Dickson RB., Salomon D. Expression of amphiregulin cripto-1, and heregulin α in human breast cancer cells. Int J Oncol 1993;2:903-11.

Kenny N, Johnson G, Selvam MP, Kim N, Qi F-F, Saeki T, Brandt R, Jones BW, Ciardiello F, Shoyab M, Plowman G, Day A, Salomon DS, Normanno N. Transforming growth factor α (TGFα) and amphiregulin (AR) as autocrine growth factors in nontransformed immortalized 184N1N4 human mammary epithelial cells. Mol Cell Diff 1993;1(2):163-84.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 09022-08 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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: G.L. Prasad — Visiting Scientist — LTIB, DCBDC, NCI

Others:

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: 1.0	PROFESSIONAL: 1.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The major goal of this research project is to understand the molecular relationship of suppression of tropomyosin (TM) expression to neoplastic transformation. Earlier work carried out in this laboratory suggested that TM suppression plays a causal role in cellular transformation. Supporting evidence for this hypothesis was obtained by restoring expression of one of the two suppressed isoforms of TM (TM1) in a v-Ki-ras-transformed cell line, DT, by means of retroviral gene transfer. Transduced cell lines of DT which express elevated levels of TM did not participate in anchorage independent growth or tumorigenesis in nude mice. Transduced TM participated in the reemergence of microfilament network to a limited degree, as evidenced by immunofluorescence and biochemical analysis. It is important to understand the molecular basis of this phenomenon before therapies for human cancers can be considered based on TM gene replacement. Normal NIH3T3 cells were also transduced with TM1 and its metabolism was studied. Results of these experiments revealed that homodimers of TM1 protein did not form stable components in the cytoskeleton in either normal or transformed cells. This may explain the partial restoration of cytoskelton observed with DT transduced lines. cell lines DT which express both the suppressed isoforms, viz., 1 and 2 are constructed and being examined.

Earlier, we have demonstrated severe deficiencies in TM expression in human breast carcinoma cell lines. In order to investigate if the observed anomalies of TM metabolism do exist in the human breast carcinoma cell lines, an isoform specific antibody has been developed.

Major Findings

We have continued studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. In an attempt to understand the critical biochemical changes in transformed cells, earlier work from our laboratory identified suppression of two isoforms ("muscle-type") of tropomyosins as the common defect in murine fibroblasts transformed by several unrelated oncogenes and chemical mutagens. Restoration of expression of these proteins was observed in "flat revertants" of *ras* transformed NIH3T3 cells. Also in normal rat kidney fibroblasts treated with transforming growth factor , expression of these two isoforms of TM is down regulated and a steep decline in their utilization into cytoskeleton precedes morphological transformation. More recent studies with human breast carcinoma cell lines have demonstrated severe derangement's in expression of "muscle-type" TMs in comparison with normal human mammary epithelial cells (HMEC). These results with different model systems have established that the defects in TM synthesis and utilization are consistently associated with cellular transformation; the disruption of microfilament architecture may be primarily due to aberrations in TM expression. This raises the possibility that TMs play a causal role in neoplastic transformation. This hypothesis was supported by our recent experiments which showed 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 growth in athymic nude 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.

Our recent efforts have been directed at further understanding of the suppression of transformed phenotype in response to elevated TM expression. The following aspects have been addressed.

Utilization TM1 in normal and transformed cells

Expression of the tropomyosin-1 isoform was enhanced by cDNA transfer in nontransformed murine 3T3 fibroblasts and also in v-Ki-*ras* transformed fibroblasts in which native tropomyosin-1 expression had been reduced and tropomyosin-2 synthesis virtually eliminated by action of the oncogene. The level of synthesis of insert-derived tropomyosin-1 was similar in normal and transformed transductants (3 - 5 times normal levels). The high level of insert tropomyosin-1 expression resulted in a considerable increase in tropomyosin-1 utilization in the cytoskeleton of transformed cells, but this still did not reach normal levels, suggesting an oncogene-related inhibition of tropomyosin- utilization. A large proportion of newly synthesized native tropomyosin-1 in normal, unmodified fibroblasts appeared in homodimers which, upon prolonged incubation were largely converted to heterodimers. Excess tropomyosin-1 derived from the inserted cDNA also appeared largely as the homodimer in both normal and transformed cells. This homodimer was utilized effectively in the formation of cytoskeletal structures but was partially converted to heterodimer by chain exchange. Under steady-state conditions, about one third of the cytoskeletal tropomyosin-1-containing dimers were homodimers, compared to about 10% in normal fibroblasts. The results show that the increased amount of tropomyosin-1 homodimer entering the cytoskeleton under conditions of tropomyosin-1 excess results in an atypical microfilament composition. The effect of this excess of tropomyosin-1 homodimers on stability or function of microfilament fibers remains to be determined. The *in vivo* results confirm findings of *in vitro* studies that the mechanisms of rapid homodimer formation and their conversion, by chain exchange, to heterodimers also occur *in vivo*.

Expression of TM1 and TM2 in DT cells

As noted above, TM1 expression does not completely correct the defect in TM expression in DT cells, because another isoform, TM2 remains virtually absent. The poor cytoskeletal utilization of enhanced levels of TM1 may be due to the absence of TM2, which is the physiological partner of TM1 in microfilament assembly. Alternatively, some specific effect of ras expression may persist in inhibiting TM utilization. To answer this question, mouse TM2 was cloned by RT-PCR technique

and its expression construct was introduced in to a DT/S cell clone through an expression plasmid. The resultant cell lines which express both TM1 and TM2 are characterized and now being examined for utilization of TM into the cytoskeleton.

Isoform specific antisera for TM

TM expression is severely impaired in the human breast carcinoma cell lines. Since the major goal of this project is to evaluate the relationship of TM in Human cancer, we would like to establish if defects of TM metabolism can be found in the clinical samples; if defects in TM expression are found we wish to test if they could be used as prognostic indicators for diagnostic purposes. A major limitation for these important goals is the lack of isoform specific antisera for TMs. By using known amino acid sequences of TMs, we have identified a polypeptide stretch that is specific for TM1 and generated antiserum against this peptide. The rationale for concentrating on TM1 is that its expression is completely extinguished in the breast carcinoma cell lines tested. This antiserum recognized specifically TM1 from fibroblasts, but not other isoforms, on western blotting. Currently we are testing this antiserum for detection of TM1 in cell lines of mammary epithelium.

Publications

Prasad GL, Fuldner RA, Cooper HL . Expression of transduced tropomyosin-1 cDNA suppresses neoplastic growth of cells transformed by *ras* oncogene. Proc Natl Acad Sci USA 1993; 90:7039-43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 CB 04829-19 LTIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
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COOPERATING UNITS (if any)

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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

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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.)

We have undertaken on going 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 specfic regions of the cellular genome in tumor DNA. In previous studies we have found LOH on chromosomes 1p, lq, 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 chromosome17p13 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 messured by BUDR incorporation. Furthermore, this association appears to primarily refect those tumors having a mutaion in either exon 5 (p=0.0002) or exon 6 (p=0.05). There is a body of evience 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 chromosome17.

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.

Our approach has been to develop high density deletion maps of the affected chromosomes in breast tumor DNAs using PCR-based polymorphic STS markers. Our markers suggest that on chromosome 1p there are at least two tumor suppressor genes located at 1p13-p21 and 1p32-pter that are affected by LOH in primary breast tumors. Similarly, five regions of chromosome 17 appear to be independently affected by LOH. Three of these regions are on the long arm of 17, located at 17q12-q21.3, 17q22, and 17q23-qter. The other two regions affected by LOH are at chromosome 17p13.1 and 17p12.3 to 17pter. BRCA1, the gene associated with familial breast cancer is located on chromosome 17q21. In sporadic breast tumors we have defined a 150kb within this region which is frequently affected by LOH. Currently we are attempting to identify the target gene within this region and determine its relationship to BRAC1.

The *TP53* gene

The *TP53* gene is located on chromosome 17p13.1 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 of121 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. 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.

The *NME1* gene

We have reported that chromosome 17q21 is also frequently affected by LOH in primary breast tumors. The *NME1* gene, located at 17q21, was affected by LOH in 64% of the informative tumors. 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* is 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 polyacryamide 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.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 05148-15 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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	Francesca Diella	Visiting Fellow	LTIB, DCBDC, NCI
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	Shukichi Miyazaki	Visiting Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
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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

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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 mammary tumor system has been useful both in the identification and characterization of genes involved in the development and transformation of mammary epithelium. In previous studies we identified and characterized the Notch related *Int*-3 gene which is frequently rearranged by MMTV in mouse mammary tumors. More recently the analysis of one of our 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 *Int*-6. It was found to be rearranged by MMTV in 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. It appears to be unrelated to any known gene in the GenBank. In tumors in which *Int*-6 is rearranged by MMTV the intergration site is within an intron and the transcriptional orientiation of the viral genome is opposite to that of the Int-6 gene. This leads to the expression of a truncated Int-6 gene product. The biological activity of the MMTV rearranged Int-6 gene is being tested. The HC11 mouse mammary epithelial cells, while maintaing certain characteristics of normal cells, are immortal. Further analysis of this cell line reveals that one allele of the *Trp*53 gene contains a microdeletion of seven condons in exon 5 and the other allele contains a missense mutation in exon6. Introduction of the wild type *trp*53 gene into these cells suppresses their grwoth. This cell line may therefore be useful in studies aimed at the cooperative interaction of mutations in mammary carcinogenesis.

Major Findings:

Characterization of *Int-3*

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. spetus*; 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. The nucleotide sequence of *Int*-6 cDNA has been determined and is unrelated to the sequence of any known gene in the GenBank. The gene is expressed in all adult tissue which have been tested, including the mammary gland. In tumors in which the gene is rearranged by MMTV the viral genome is integrated within an intron in the opposite transcriptional orientation. This leads to the expression of a chimeric MMTV-*Int*-6 RNA species, which encodes a truncated I*nt*-6 protein. We are currently testing the biological activity of these novel *Int*-6 rearranged genes.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 05216-23 LTIB

PERIOD COVERED: October 1, 1993 to September 30, 1994

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)

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Cellular Biochemistry Section

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

TOTAL STAFF YEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)
[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither
[ ] (a1) Minors
[ ] (a2) Interviews

B

SUMMARY OF WORK (Use standard unreduced 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-Cl-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 RIα with up-regulation of RIIβ 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 RIα and RIIβ regulatory subunits of cAMP-dependent protein kinase, have opposite roles in cell growth and differentiation; RIα being growth stimulatory while RIIβ is a growth-inhibitory and differentiation-inducing protein. As RIα 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-Cl-cAMP and RIα antisense oligodeoxynucleotide, those that effectively down-regulate RI and up-regulate RIIβ, provide new approaches toward differentiation therapy and chemoprevention of cancer. 8-Cl-cAMP is now in Phase I clinical studies at several foreign Institutes.

Major Findings

An antisense oligodeoxynucleotide that depletes RIα subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. Enhanced expression of the RIα subunit of cyclic AMP-dependent protein kinase type I has been correlated with cancer cell growth. We provide evidence that RIα 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 RIα 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 RIα antisense oligomer correlated with a decrease in the RIα mRNA and protein levels and with an increase in RIIβ (the regulatory subunit of protein kinase type II) expression. The growth inhibition was abolished, however, when cells were exposed simultaneously to both RIα and RIIβ antisense S-oligodeoxynucleotides. The RIIβ antisense S-oligodeoxynucleotide alone, exhibiting suppression of RIIβ along with enhancement of RIβ expression, led to slight stimulation of cell growth. These results demonstrate that two isoforms of cyclic AMP receptor proteins, RIα and RIIβ are reciprocally related in the growth control of cancer cells and that the RIα antisense oligodeoxynucleotide, which efficiently depletes the growth stimulatory RIα, 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 RIα of PKA is consistently observed in MDR cancer cell lines as compared to non-MDR parent cell lines. 8-Cl-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 RIα 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‡ by immunoblotting and C by PKA activity ratio, which measures the degree of free C subunit release. 8-Cl-cAMP treatment sharply reduced RIα 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 RIα antisense S-oligonucleotide (6 μM) for 4-5 days brought about marked growth inhibition. RIIβ antisense or random S-oligomers had no effect. The effect of RIα antisense oligonucleotide on MDR-1 expression is being assessed. These results suggest that RIα but not C subunit of PKA may by causally related to the multidrug resistance of cancer cells.

Different expression of mRNA for RIα subunit of cAMP-dependent protein kinase (PKA) between breast and colorectal carcinomas and normal counterparts. Enhanced expression of type I regulatory subunits (RIα) 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 RIα mRNA were detected, whereas in normal breast, only 4.4 kb RIα mRNA was detected. The expression of four species of RIα 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 RIα 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 RIα mRNA were detected as compared to the adjacent normal colorectal tissues. Studies are underway to determine whether RIα mRNA expression can be of value for the diagnosis and prognosis of breast and colon cancers.

Retroviral vector-mediated overexpression of the RIIβ subunit of the cAMP-dependent protein kinase induces differentiation in human leukemia cells and reverts the transformed phenotype of mouse fibroblasts. We have recently shown, using antisense strategy, that the RIIβ regulatory subunit of cAMP-dependent protein kinase is essential for cAMP-induced growth inhibition and differentiation of HL-60 human leukemia cells. We constructed a retroviral vector for RIIβ (MT-RIIβ) by inserting human RIIβ cDNA into the OT1521 retroviral vector plasmid that contains an internal mouse metallothionein-1 promoter and a neomycin resistance gene. The PA137 packaging cell line was then transfected with MT-RIIβ plasmid to produce the amphotrophic stock of MT-RIIβ retroviral vector. The infection with MT-RIIβ and treatment with $CdCl_2$ brought about growth arrest in HL-60 human leukemia and Ki-ras-transformed NIH 3T3 clone DT cells in monolayer culture with no sign of toxicity. The growth inhibition correlated with the expression of RIIβ and accompanied changes in cell morphology; cells became flat exhibiting enlarged cytoplasm. The growth of these cells in semisolid medium (anchorage-independent growth) was almost completely suppressed. In contrast, overexpression of the RIα subunit of protein kinase enhanced the cell proliferation in DT cells. The MTRIIβ infected cells exhibited an increases sensitivity toward treatment with cAMP analogs, such as 8-Cl-cAMP and N6-benzyl-cAMP, as compared with the parental noninfected cells. In MTRIIβ HL-60 cells, N6-benzyl-cAMP treatment greatly enhanced the expression of monocytic surface markers. These results suggest that the RIIβ cAMP receptor, by binding to its ligand, cAMP, acts as a tumor suppressor protein exerting growth inhibition, differentiation, and reverse transformation.

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Bold RJ, Warren R, Ishizuka J, Cho-Chung YS, Townsend CM, Thompson JC. Experimental gene therapy of human colon cancer. Surgery (in press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 08281-12 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Cellular Biochemistry Section

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

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

B

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

The striking growth inhibitory effect of 8-Cl-cAMP has been related to its selective binding and activation of protein kinase isozymes: It binds to RII 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-Cl-cAMP brought about various effects among the cell lines tested, including the suppression of oncogenes and transforming growth factor a (TGFα), and morphological changes, differentiation, and reverse transformation. Despite the appearance of markers of mature phenotype and definitive growth arrest, the 8-Cl-cAMP-treated leukemic cells exhibited no change in the cell cycle phase. 8-Cl-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-Cl-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-Cl-cAMP include a rapid nuclear translocation of RIIβ, and such translocation of RIIβ into the nucleus correlates with an increase n 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 RIIβ 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 autophorylation 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 RIIβ of introduced into RIα 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-Cl-cAMP induces truncation and down-regulation of the RIα subunit and up- regulation of the RIIβ subunit of cAMP-dependent protein kinase leading to type II holoenzyme-dependent growth inhibition and differentiation of HL-60 leukemia cells. 8-Cl-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-Cl-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-Cl-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-Cl-cAMP (5 μM, 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-Cl-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-Cl-cAMP treatment was observed in cells infected with the human RIIβ 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-Cl-cAMP treatment, RIIβ 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 RIIβ gene. We identified the 48-kDa RI as RIα, the 50-kDa RII as RIIα, the 52-kDa RII as RIIβ, and the 54-kDa RII as the phosphorylated form of either the RIIα or RIIβ subunit. *In vivo* labeling experiments using [^{3}H]8-Cl-cAMP demonstrated that 8-Cl-cAMP enters cells and binds to both PKI and PKII. The [^{3}H]8-Cl-cAMP binding profile of HPLC showed that 8-Cl-cAMP selectively down-regulates PKI and up-regulates PKII, and such an effect of 8-Cl-cAMP is mimicked by exposing cells to RIα antisense oligodeoxynucleotide, which suppresses RIα and enhances RIIβ expression. Dot-blot hybridization analysis demonstrated that both 8-Cl-cAMP treatment (5 μM, 6 h) and RIIβ retroviral vector infection elicited a marked induction of RIIβ and Cα mRNA with little or no change in RIα and RIIα mRNA. These results suggest that the RIα and RIIα 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-Cl-cAMP, a site-selective cAMP analog as a novel agent that inhibits the promoter activity of multidruq-resistance (MDR-1) gene. We have shown previously that 8-Cl-cAMP inhibits the growth of multidrug resistant (MDR) cell lines and down-regulates p-glycoprotein. In this study, we demonstrate that 8-Cl-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-Cl-cAMP. 8-Cl-cAMP treatment (2.5μM) 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-Cl-adenosine, did not mimic the effect of 8-Cl-cAMP, indicating that the CAT activity inhibition was due to the intact molecule of 8-Cl-cAMP, rather than due to its metabolites. Since the mechanism of 8-Cl-cAMP action involves upregulation and nuclear translocation of type II regulatory (RIIβ) subunit of cAMP-dependent protein kinase, we examined the effect of RIIβ gene by co-transfecting it with MDRCAT gene. It was found that RIIβ gene inhibits the CAT activity mimicking the effect of 8-Cl-cAMP. Thus, RIIβ may be the negative regulator of MDR promoter.

Introducing an autophosphosrlation site mutation in the RIIβ regulatory subunit of cAMP-dependent protein kinase abolishes the RIIβ-mediated regulatory function. We have previously shown by the use of site -selective cAMP analogs and antisense strategy that the RIα subunit of protein kinase is a growth stimulatory protein wheras the RIIβ subunit is a growth inhibitory and differentiation-inducing protein. The RIIβ regulatory subunit has an autophosphorylation site, while the RIα subunit has a pseudophosphorylation site. In order to determine the functional significance of regulatory subunit autophosphorylation, an autophosphorylation site mutation was introduced into RIIβ using site-directed mutagenesis. The serine 114 of human RIIβ was replaced with alanine. We then constructed retroviral vectors for the wild type and mutant RIIβ in the OT1521 retroviral vectorplasmid that contains an internal mouse metallohionine-1 promoter and a neomycin resistance gene. Transfection with wild type RIIβ retroviral vector and treatment with ZnSO4 brought about growth-inhibition and changes in cell morphology in LS-174T colon carcinoma and Ki-ras-transformed NIH3T3 cells (clone DT), and these cells did not produce colonies in soft agar. In contrast, cells transfected with the mutant RIIβ gene upon treatment with ZnSO4 continued to grow at the same rate as that of control cells (untransfected), and were able to form large large colonies in soft agar. Northern and Western blots demonstrated high expression of both RIIβ and mutant RIIβ genes in transfected cells. The reduced cAMP-inhibitory effect on the EGF-activated MAP kinase observed in the mutant RIIβ-tranfectant is correlated with the lack of growth regulatory function of mutant RIIβ.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 04848-22 LTIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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

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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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☐ (a1) Minors
☐ (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 *rsu*-1, is a novel gene which specifically suppresses v-Ki-*ras* and v-Ha-*ras* transformation of fibroblasts and epithelial cells. The *rsu*-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 *rsu*-1 may physically associate with *ras* p21or ras interacting molecules and alter Ras signal transduction in this way. In non-transformed cells p33 *rsu*-1 is phosphorylated in response to stimuli which activate Ras signal transduction pathways. Phosphorylation of p33 Rsu-1 is dependent on activation of Ras in these pathways, indicting Rsu-1 is part of a "downstream" effector or regulators pathway. *rsu*-1 is a phylogenetically highly conserved molecule; cloning and sequencing of the human *rsu*-1 cDNA revealed that the human *rsu*-1 protein is 96% homologous to the mouse rsu-1. Screening of over 100 cell lines and tissue, both human and rodent, revealed that *rsu*-1 RNA expression is ubiquitous. The human *rsu*-1 gene has been localized to human chromosome 10p13, a region of chromosome 10 frequently deleted in high grade glioblastoma. Our current efforts are aimed at elucidating the mechanisms by which this molecule disrupts *ras* signal transduction in vitro and determining if the *rsu*-1 locus is disrupted in humanglioblastomas and other 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.

The better studied of the recovered clones is a novel gene referred to as *rsu-1*. The introduction of *rsu-1* cDNA into DT cells suppressed the growth of that cell line in agar by 30-75% and yielded phenotypically flat revertants. *rsu-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 *rsu-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 *rsu-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 adenylyl cyclase. The homology is confined to a series of leucine based repeats 23 amino acids in length which are necessary for the activation of adenylyl cyclase by *ras* in Sa. *cerevisiae*. This homology, in conjunction with the *ras* suppressor activity, suggests that *rsu-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 *rsu-1* also be phylogenetically highly conserved. Cloning and sequencing the human *rsu-1* cDNA revealed that it too encodes a 277 amino acid protein which is 96% homologous to the mouse *rsu-1*. Western blotting and immunoprecipitation using anti *rsu-1* antibody detect a 33kd protein in human as well as rodent cells. Southern blotting revealed that *rsu-1* is a single copy gene, and that human and mouse *rsu-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 *rsu-1* RNA. We have localized the *rsu-1* locus to human chromosome 10p13 and identified human tumor cell lines containing low levels of *rsu-1* RNA and protein. The most interesting of these cell lines is a human glioblastoma multiforme derived cell line, U251, which contains a low level of *rsu-1* specific RNA but no detectable *rsu-1* protein. There are numerous reports of chromosome 10 deletion during the transition from high grade astrocytoma to glioblastoma multiforme; the losses include the region to which *rsu-1* has been localized (10p13). This suggests that *rsu-1* may serve as a tumor suppressor in glioblastoma progression. To test this, the U251 cell line was reconstructed with a *rsu-1* expression plasmid to express *rsu-1* protein under the control of an inducible plasmid promotor. Induction of *rsu-1* expression increases the doubling time of the U251 cells by >50%. Experiments testing the effect of *rsu-1* on suppression of U251 tumor formation in nude mice are underway.

p33 *rsu-1* is rapidly phosphorylated in response on serine and threonine residues in response to growth factors (EGF, NGF, IGF-1) and other stimulators (TPA, serum) which activate *ras* signal tranduction pathways. In addition, in cells containing v-Ha-*ras* under the control of an inducilbe promotor, induction results in p33 *rsu-1* phosphorylation. In cells containing dominant negative (Asn17) v-Ha-*ras* in an inducible form, induction inhibits p33 *rsu-1* phosphorylation in response to growth factors. These results indicate that *rsu-1* lies "downstream" of *ras* in signal transduction pathways in an effector or regulatory capacity. Using inhibitors of phosphorylation

specific for individual or categories of kinases. We are determining the nature of the phosphorylation reaction involving *rsu-1.* In addition, the isolation and identification of proteins binding to or associating with *ras*-1 is proceeding utilizing yeast dual hybrid systems. By employing these two methods the nature of *rsu-1* phosphoylation reaction and its role in signal transduction should become evident. 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

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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Hormones, Antihormones and Growth Factors in Mammary Development and Tumorigenesis

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LAB/BRANCH: Laboratory of Tumor Immunology and Biology

SECTION: Molecular and Cellular Endocrinology

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TOTAL MAN-YEARS: 4.75	PROFESSIONAL: 3.75	OTHER: 1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The 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 (Prl), estrogens, and progesterone, with recent work also examining how epidermal growth factor (EGF), and transforming growth factors α and β 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, Prl and glucocorticoids, EGF or TGF-α can promote full lobulo-alveolar development in vitro. High concentrations of the somatogenic hormone, growth hormone, can substitute for Prl in this system. This effect is not mediated through insulin-like growth factor I. Lobulo-alveolar development in vitro is not inhibited by TGF-β; casein synthesis is. In addition, TGF-β dramatically inhibits ductal outgrowth by epithelial cells transduced with the human TGF-β1 gene and transplanted back into the cleared mammary fat pad. Prl induced growth of the mouse mammary epithelial cell, NOG-8, involves activation of protein kinase C (PKC). Prl induces translocation of the PKC from cytosol to the membranes within 10 min. of exposure to the hormone. In addition, mitogen-activated protein (MAP) kinases are activated within 5 min. by Prl in NOG-8, T47D and PC-12 cells. When NOG-8 cells are transformed with *ras* oncogene they lose the ability to bind Prl when grown in the presence of charcoal stripped serum. This effect is reversed by the addition of Prl to the culture medium, or by introducing the *rsp-1 ras* suppressor gene. We have explored the relationship of a membrane associated antilactogen binding site (ALBS) to the Prl receptor on human breast cancer cell growth in culture. Prl induced growth of human breast cancer cells can be blocked by non-steroidal antiestrogens such as tamoxifen (TAM). This action is through the ALBS which appears to be intimately associated with the Prl receptor. These studies are greatly aided by use of monoclonal antibody B6.2 which is the first known such antibody to recognize the human Prl receptor. The antiprolactin action of TAM, working through the ALBS, may have important clinical implications. Finally, we have begun to explore the effects of neonatal exposure to TAM on mammary gland development and tumorigenesis and find that the normal mammary gland is poorly developed in the TAM treated mice and there are more pre-neoplastic hyperplastic alveolar nodules.

Major Findings

In addition to the classical hormones such as insulin (I), corticoids (A and H), prolactin (Prl), 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. Withdrawal of these hormones and growth factors results in programmed cell death (apoptosis) by the mammary gland.

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-β mRNA. Effects on DNA synthesis and EGF receptors are primarily due to P. E increases TGF-α 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 Prl in the medium. At high concentrations, growth hormone (GH) replaced Prl for lobulo-alveolar development as well as casein gene expression. Addition of EGF to the cultures with either Prl or GH resulted in maximal development. Insulin-like growth factor I did not substitute for Prl or GH nor did it substitute for insulin in tissue maintenance even in the presence of EGF. GH may act by increasing the number of prolactin receptors on the cell surface. Mammary glands make their own prolactin.

Previously we had shown that local, *in vivo*, induction of lobulo-alveolar development occurs in mouse mammary glands exposed to EGF or TGF-α 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-β_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-β_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 $CdCl_2$ 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 $ZnSO_4$ 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-β_1 construct had truncated or no ducts and abnormal, enlarged terminal end buds.

Prl-induced growth of the normal mouse mammary NOG-8 cells involves activation of protein kinase C (PKC). Treatment of cells with either Prl or the phorbol ester, PMA, or both together, gave an 8 fold increase in PKC activity with maximal stimulation occurring within 10 minutes of Prl treatment. H7, a potent PKC inhibitor, completely inhibited the hormone induced enzyme activity. Subsequent increases in cell numbers were also effectively blocked by H7. Prl treatment translocated the PKC activity from the cytosol to the plasma membranes. Within 1 to 5 minutes of exposure to Prl, NOG-8, T47D and PC-12 pheochromocytoma cells

demonstrate activation of the mitogen-activated protein (MAP) kinase signal transduction pathway. This activation is blocked by genestein and tyrphostin, known tyrosine kinase inhibitors.

NOG-8 cells binds Prl 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. Prl binding was restored by returning the cells to FCS or by supplementing the CSS with Prl. 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-α 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 $CdCl_2$ 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 $p21^{ras}$ 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 ^{3}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_1 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_1. 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.
The stably transfected cells containing the Prl receptor also acquire TAM sensitivity. This proves that the ALBS is on the Prl receptor.

Neonatal exposure to estrogens results in reproductive tract abnormalities in the adult animal. We are looking at the effects of neonatal exposure to TAM and the related antiestrogen , nafoxidine, on mammary gland development. In the C3H/HeN MTV+ mouse, the mammary glands are poorly developed at three

months of age neonatal exposure to either antiestrogen. The number of pre-neoplastic hyperplastic alveolar nodules is elevated in the exposed animals.

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-β. Apoptosis during involution both in vivo and in vitro will be examined by DNA ladder formation, nuclear staining and expression of specific genes. We will use the transplantation system to examine the effects of different mitogens during transfection on subsequent mammary cell outgrowth. Histological examination of the bulbous end buds which occur under the influence of TGF-β_1 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. The ability of TAM and DES to affect mammary tumor development in C3H/HeN (MTV+) mice and normal development of glands in Balb/c mice will be investigated.

Publications

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Banerjee R, Cutler ML, Vonderhaar BK. Repression of lactogen binding in NOG-8 mammary epithelial cells by V-*Ras*. Mole and Cell Diff 1993;1: 215-31.

Smith JJ, Capuco AV, Mather IH, Vonderhaar BK. Ruminants express a prolactin receptor of M_r 33,000-36,000 in the mammary gland throughout pregnancy and lactation. J. Endocrinol 1993;139: 37-49.

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Banerjee R, Ginsburg E, Vonderhaar BK. Characterization of a monoclonal antibody against human prolactin receptors. Int. J. Cancer 1993; 55: 712-21.

Banerjee R, Biswas R, Vonderhaar BK. Characteristics of the antilactogen binding site in mammary gland membranes. Mol Cell Endocrinol 1993; 98:1-8.

SUMMARY STATEMENT

VARMUS LABORATORY

DCBDC, NCI

October 1, 1993 to September 30, 1994

The Varmus Laboratory uses molecular and genetic approaches to understand the normal and pathogenic roles of genes implicated in human and animal cancers. It is convenient to consider our activities in three categories:

(1) The src gene family. We are currently emphasizing the use of mice with targeted mutations in the four src-like genes expressed in the monomyelocytic lineage--src, fgr, hck, and lyn--to explore the normal functions of these genes. At least three issues are under study: What is responsible for the loss of natural immunity in mice lacking both hck and fgr? Can we protect src-deficient mice from osteopetrosis by expressing a src transgene in the macrophage lineage? What accounts for the defect in hematopoiesis observed in mice deprived of both hck and src? In pursuing these questions, we are also making use of recent findings that suggest that some normal function of src protein can be attributed to the aminoterminal half, rather than to the kinase domain.

(2) The Wnt gene family. A variety of approaches are being utilized to seek receptors for the secretory glycoproteins encoded by Wnt genes--genes that have been implicated in a broad collection of developmental events, as well as tumor formation. These studies include efforts to bind Wnt fusion proteins to the surface of cells expressing receptors and structural and functional screens of cDNA libraries, using assays in mammalian cells and frog embryos. In conjunction with these efforts, we are examining the potential signalling pathways activated by Wnt proteins and some candidate receptor genes that have been recently isolated.

(3) Mammary carcinogenesis. Transgenic mice that express the Wnt-1 gene in the mammary gland and develop mammary hyperplasia and malignant carcinoma are being used to study the multi-step process required for the full oncogenic phenotype. An activated Wnt gene can collaborate with activated FGF genes or with inactivated p53 genes to accelerate tumorigenesis; other collaborating genes are being sought by genetic crosses and other methods, including insertional mutagenesis with mouse mammary tumor virus. We are also using analysis of karyotypes, specific genes, and the physiological behavior of mammary cells to understand the contributions made by each event in tumorigenesis. Furthermore, we are examining the role of tumor immunity in the generation of these neoplasms.

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

PROJECT NUMBER
Z01 CB 09426-01 VL

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Normal and Oncogenic Functions of Src and Wnt Gene Families

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

PI:	H. E. Varmus	Chief, Varmus Laboratory	VL, NCI
Others:	M. Chamorro	IPA Investigator	VL, NCI
	L. Godley	Pre-doctoral IRTA Fellow	VL, NCI
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COOPERATING UNITS (if any)

I.G. Dawid, LMG, NICHD; L. Donehower, Baylor University; J. Gray, UCSF; T. Jacks, MIT; M.J. Lenardo, NIAID; R. Nusse, Stanford University

LAB/BRANCH
Varmus Laboratory

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

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.)

This laboratory uses molecular and genetic approaches to understand the normal and pathogenic roles of genes implicated in human and animal cancers. It is convenient to consider the activities in three categories: 1) The src gene family. We are currently emphasizing the use of mice with targeted mutations in the four src-like genes expressed in the monomyelocytic lineage--src, fgr, hck, and lyn--to explore the normal functions of these genes. At least three issues are under study: What is responsible for the loss of natural immunity in mice lacking both hck and fgr? Can we protect src-deficient mice from osteopetrosis by expressing a src transgene in the macrophage lineage? What accounts for the defect in hematopoiesis observed in mice deprived of both hck and src? 2) The Wnt gene family. A variety of approaches are being utilized to seek receptors for the secretory glycoproteins encoded by Wnt genes--genes that have been implicated in a broad collection of developmental events, as well as tumor formation. These studies include efforts to bind Wnt fusion proteins to the surface of cells expressing receptors and structural and functional screens of cDNA libraries, using assays in mammalian cells and frog embryos. In conjunction with these efforts, they are examining the potential signalling pathways activated by Wnt proteins and some candidate receptor genes that have been recently isolated. 3) Mammary carcinogenesis. Transgenic mice that express the Wnt-1 gene in the mammary gland and develop mammary hyperplasia and malignant carcinoma are being used to study the multi-step process required for the full oncogenic phenotype. An activated Wnt gene can collaborate with activated FGF genes or with inactivated p53 genes to accelerate tumorigenesis; other collaborating genes are being sought by genetic crosses and other methods, including insertional mutagenesis with mouse mammary tumor virus. We are also using analysis of karyotypes, specific genes, and the physiological behavior of mammary cells to understand the contributions made by each event in tumorigenesis.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBDC, NCI
October 1, 1993 through September 30, 1994

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):

The major area of study of this laboratory is the role of the epidermis as an immunological organ. We are continuing our studies of the very earliest events which occur during allergic contact dermatitis which we use as a paradigm for studying the skin immune system. Within 24 hours after exposure of epidermis to haptens, 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. There is an almost immediate upregulation of IL-1 β mRNA from Langerhans cells after hapten application, and later, an upregulation of IL-10 mRNA by keratinocytes. These cytokines exert functional effects on the skin, both *in vivo* and *in vitro*.

To better understand the nature of the precise epitopes generated after hapten-Langerhans cell interaction, we have begun assessing the ability of TNP-modified I-A binding peptides to activate hapten-specific $CD4^+$ T cells. Using Langerhans cells as antigen presenting cells, $I\text{-}A^k$ restricted TNP-specific $CD4^+$ T cells proliferated in the presence of the 10mer synthetic peptide hen egg lysozyme (HEL) 52-61 derivatized with TNP in position 56 (TNP-K56), and less so when TNP occupied positions 53 (TNP-K53) or 59 (TNP-K59). The requirement for direct interaction between TNP-peptides and MHC class II molecules for T cell recognition was also confirmed by the absence of response of haptenated peptides using BALB/c ($H\text{-}2^d$) $CD4^+$ T responder cells (from sensitized mice). Our results indicate that hapten-modified MHC class II binding peptides generate epitopes recognized by hapten-specific $CD4^+$ T cells and that precise positioning of hapten molecules is required for optimal $CD4^+$ T cell recognition. These finding provide insight into how haptens are recognized by T cells in contact sensitivity and should facilitate the study and design of specific therapies for the manipulation of hapten-specific $CD4^+$ T cell responses.

We are continuing our studies of the role of Langerhans cell in HIV. Although HIV reportedly infects Langerhans cells, the functional status of Langerhans cell in HIV infection is unknown. We studied Langerhans cell function and monocyte/macrophage function in 21 HIV-seropositive (HIV^+) and 21 HIV-

seronegative (HIV^-) volunteers, including 3 monozygotic twin pairs discordant for HIV serology. Langerhans cells from HIV^+ individuals were quantitatively normal and expressed normal levels of HLA-DR. However, both Langerhans cells and monocytes from HIV^+ individuals stimulated allogeneic T cells less well compared to control APC. In addition, decreased protein antigen- and mitogen-induced T cell responsiveness was observed in HIV^+ individuals (particularly AIDS patients), using either autologous Langerhans cells or autologous monocytes as antigen presenting cells. This was undoubtedly due to a defective T cell response because Langerhans cells and monocytes isolated from the HIV^+ twins were able to present protein antigen normally to HIV-uninfected syngeneic T cells. Thus, although alloantigen presentation by Langerhans cells and monocytes from HIV^+ individuals was impaired, results of twin studies indicated that protein antigen presentation by these cells was intact. We conclude that immunologic dysregulation in early and intermediate stages of HIV disease does not reflect marked functional impairment of antigen presenting cells, but may be secondary to T cell dysfunction alone. (Time devoted to AIDS is 25%)

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, as well as in leukocyte-epithelial cell adhesion. During the past year it has been demonstrated that the expression of the homophilic adhesion molecule E-cadherin appears to be a general feature of Langerhans cells, since human as well as mouse epidermal Langerhans cells express E-cadherin. A survey of murine lymphoid dendritic cells has determined that Langerhans cells are unique among dendritic cells in their ability to express high levels of E-cadherin. Dendritic cells from spleen and gut-associated lymph nodes failed to express E-cadherin. Low levels of E-cadherin are expressed by dendritic cells isolated from skin-associated lymph nodes, however, consistent with the proposed lineage relationship between these cells and epidermal Langerhans cells. A subpopulation of thymic dendritic cells may also express E-cadherin.

In an attempt to develop a model system that will permit studies of mechanisms that regulate the level of expression and affinity of E-cadherin molecules on the surfaces of Langerhans cells or related cells, dendritic cells have been propagated from murine blood, bone marrow and skin in vitro. Cells grown from blood and bone marrow in media supplemented with GM-CSF express differentiation antigens typical of several kinds of dendritic cells, and also express E-cadherin. Based on the surface antigens that they express, dendritic cells obtained from cultures of blood and bone marrow are not identical to any single population of tissue dendritic cells including epidermal Langerhans cells, however. In addition, although they express E-cadherin, these dendritic cells do not adhere avidly to keratinocytes or other cells that express E-cadherin. Thus, it is not obvious that dendritic cells propagated from blood or bone are suitable for future studies. Leukocytes from fetal skin have also been expanded in GM-CSF supplemented media with the expectation that these cells might resemble Langerhans cells more closely than cells obtained from blood or bone marrow. Leukocytes propagated from fetal skin exhibit a uniform surface phenotype that is identical to that of epidermal Langerhans cells, with the important exception that most fetal skin leukocytes do not express class II MHC antigens. However, co-culturing fetal

skin leukocytes in GM-CSF and IFN-γ results in expression of cell surface class II MHC antigens, and in acquisition of enhanced accessory cell activity. To affirm that these cells represent an appropriate model system, it is important to determine if these cells can give rise to Langerhans cells in vivo. Whether or not leukocytes expanded from fetal skin can give rise to Langerhans cells will be tested by transferring them into MHC-disparate immunodeficient mice and assaying their ability to take up residence in skin, express class II MHC antigens and assume the appropriate morphology. Preliminary results suggest that leukocytes expanded from C57BL/6 fetal skin have the capacity to become Langerhans cell-like after engaftment onto BALB/cnu/nu mice in combination with BALB/c keratinocytes and fibroblasts. The ability of leukocytes propagated from fetal skin to evolve into Langerhans cells after subcutaneous and systemic administration will also be 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. Using immunochemical and molecular biologic means, the following antigens have been defined: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). This has allowed the study of their cell biologic function. BP antigen 1 (BPAG1) is in the plaque of the hemidesmosome. Molecular cloning of the full length coding sequence for BPAG1 indicates a similar domain structure to desmoplakin I, a desmosomal plaque protein. Current studies are aimed at determining the function of various subdomains of BPAG1 by transfecting cDNA that encode them into eukaryotic cells. The deduced amino acid sequence of PV antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules and is closely related to the PF antigen. PV antigen was localized ultrastructurally to the desmosome.

Current studies are also aimed at dissecting the functions of various extracellular and intracellular domains of PV antigen in cell adhesion and in binding to molecules (e.g. plakoglobin) in the desmosomal plaque. Transfection studies of mouse L cells indicate that the extracellular domain of PV antigen mediates weak homophilic adhesion. Studies with the cytoplasmic domain of PV antigen are being used to determine which domains are necessary for plakoglobin binding. Initial studies suggest that the ICS (intracytoplasmic cadherin-like segment) is necessary. Finally, transfection studies with a chimeric E-cadherin/PV antigen molecule indicate that the cytoplasmic domain of PV antigen can confer adhesion ability on the extracellular domain of E-cadherin without binding cytoplasmic catenins, previously thought to be essential for adhesion function.

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.

An extensive collaboration with Sherri Bale, Peter Steinert, and John Compton from the Lab. of Skin Biology has resulted in an effort to better characterize clinical features and to map the genes for the disorders of cornification. The group has focused on epidermolytic hyperkeratosis (EHK), recessive ichthyoses, and Darier's disease. Linkage analysis in one family with 20 members affected with EHK 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 54 EHK patients and their unaffected relatives from 22 families have been studied. There is striking heterogeneity in clinical presentation between families with EHK. Six clinical phenotypes have been distinguished on the basis of palm/sole hyperkeratosis, character and extent of scale, and presence of erythroderma. To date, mutations have been identified in 12 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. Interestingly, the same arginine to histidine mutation in keratin 10 has been found in different families with three distinct clinical phenotypes. This finding demonstrates that the specific keratin mutation is insufficient to determine the complete clinical phenotype. Further correlation of these clinical phenotypes and mutations will lead to a better understanding of the relationship between intermediate filament structure and function, and the maintenance of epidermal integrity.

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 neurodengeneration. Collaboration with Drs. Katherine K. Sanford and Ram Parshad (Department of Pathology, Howard University College of Medicine) has resulted in the development of cytogenetic tests which show very large differences in radiation-induced chromatid aberrations between normal and Alzheimer disease (AD) cells. In at least 14 of the 16 normal fibroblast lines studied, addition of caffeine (which blocks postreplication DNA repair) led to no increase in chromatid aberration frequency (CAF). However, cells from all 10 donors who were affected with Alzheimer disease in a large Canadian family and whose fibroblasts were available had a marked increase in their CAF. It appears that the patients' cells, in contrast to the normal cells, had not repaired during G_1 all the fluorescent light-induced DNA damage. The skin biopsies from three of these 10 donors were obtained years before the donors became symptomatic, documenting the presence of the DNA-repair defect in cells obtained from the donors prior to the onset of their neurologic symptoms.

Eleven asymptomatic sons and daughters of some of the ten symptomatic Canadian patients have also been studied. Each of these offspring was at a 50% risk of having inherited the disease-producing AD gene in this family. Of these 11 donors, 8 had CAF values with caffeine well in the range given by patients with AD. It is highly likely that these 8 donors will eventually become symptomatic. Several of them were less than 32 years of age at the time of

skin biopsy, suggesting that inheritance of the abnormal AD gene is manifested by the test in cells obtained at least two decades before the age of 52 years, when the disease typically produces symptoms in this family. Of the three at-risk donors who had low CAF values in the presence of caffeine, one died of cancer at the age of 49 years, and an autopsy of her brain showed no evidence to support a diagnosis of AD, indicating, therefore, that she had probably not inherited the disease-producing gene. The prediction is that the two other at-risk donors who also had low CAF values will not develop AD.

A cytogenetic test for AD using PHA-stimulated peripheral blood lymphocytes is currently being developed. Such a test would be quicker and easier to conduct than the current test using skin fibroblasts.

Ex Vivo and In Vivo Manipulations of Keratinocyte Gene Expression
(Dr. Jonathan Vogel):

The goal of this laboratory is to stably introduce and express foreign genes into keratinocytes of miniature swine epidermis. Dr. Vogel has successfully used both an in vivo approach and a ex vivo approach to introduce genes into miniature swine epidermis. The in vivo approach directly introduces the gene into epidermis while in the ex vivo approach, keratinocytes or skin organ cultures are isolated, the desired gene is inserted while in tissue culture, and the genetically modified keratinocytes are grafted back onto the donor. Selection for keratinocyte stem cells or early precursors which have the desired gene integrated into their genome is an important feature of this approach. For the purpose of both in vivo and ex vivo selection, the indicator gene β-galactosidase (βGal) has been linked to selectable marker genes which provide resistance to toxic agents such as G41-β (Geneticin), Hygromycin, and the multi-drug resistant (MDR) gene agents colchicine and vincristine. A novel in vivo approach has been developed for directly introducing and expressing the βGal indicator gene in epidermis by injecting naked plasmid DNA into the dermis underlying the epidermis. The naked DNA traverses the dermal-epidermal junction and is absorbed and expressed by keratinocytes. Colorimetric histochemical assays reveal uptake and expression of βGal plasmid DNA in keratinocytes throughout the epidermis at all stages of differentiation. A quantitative assay for βGal enzyme expression in epidermis shows that a dose response relationship exists for increasing concentrations of injected naked plasmid DNA. The presence of βGal enzyme can be demonstrated for 3 weeks following injection, but at later time points the stable enzyme is only present in the outer differentiated keratinocyte layers. Analysis of βGal RNA expression in epidermis shows expression for only 1 week suggesting that the stable βGal enzyme can persist in the epidermis even though no longer actively expressed. Topical application of the selecting agents G418 (Geneticin) and Hygromycin has not been successful for in vivo selection of keratinocytes with integrated plasmid DNA. Colchicine and vincristine appear to be superior for in vivo selection following topical application.

Ex vivo approaches utilizing selection have also been devised. The MDR gene has been transduced into primary keratinocytes using defective retroviral vectors and keratinocytes expressing the MDR gene have been selected in vitro by using colchicine and vincristine. Grafting of these keratinocytes back onto the donor miniature swine is currently being attempted. Ex vivo organ cultures of skin, which have re-epithelializing keratinocytes, have also been

both transfected and transduced with liposome-coated plasmid DNA and combinations of retroviruses respectively. These organ cultures can be selected and grafted back onto the donor with subsequent topical selection with colchicine or vincristine. The main advantage of the ex vivo approach is that a much higher percentage of the keratinocytes have the desired gene stably integrated into the genome, ensuring persistent expression.

Mechanisms of Keratinocyte Adhesion to Epidermal Basement Membrane (Dr. Kim Yancey):

The specific goal of the laboratory is to define molecules that promote adhesion of human keratinocytes to epidermal basement membrane. This fundamental issue is addressed in both clinical and basic investigative studies. In the former, patients with acquired autoimmune and inherited subepithelial bullous diseases are studied. By defining and characterizing molecules targeted by autoantibodies in patients with acquired autoimmune bullous diseases, knowledge is gained about important structural proteins in skin as well as disease pathophysiology. The laboratory has a specific interest in diseases in which patients have autoantibodies directed against bullous pemphigoid antigens 1 and 2, laminin 5 (also called kalinin or nicein), type VII collagen, or other yet-to-be-defined molecules. Similarly, by identifying proteins that are defectively expressed in the skin of patients with inherited subepithelial bullous diseases, the laboratory hopes to develop an understanding about the key role that such proteins play in epidermal homeostasis. There is specific interest in various forms of junctional epidermolysis bullosa, inherited subepithelial bullous diseases suspected to be associated with mutations in proteins that promote adhesion of basal keratinocytes to epidermal basement membrane. The identification of defective structural proteins in the skin of patients with such inherited bullous diseases will facilitate the identification of mutations in genes encoding these proteins, the prenatal diagnosis of these disorders, and their treatment (as technology to introduce exogenous genes into human keratinocytes is developed). Building on findings in these clinical investigations, the laboratory isolates native and recombinant forms of keratinocyte adhesion molecules to investigate their interactions with each other as well as with proteins in extracellular matrix. The goal of these in vitro studies is to further our understanding of epidermal morphogenesis, homeostasis, and differentiation.

Studies of patients with anti-epiligrin autoantibodies have been extended by characterizing additional patients with similar clinical and immunopathological findings (i.e., cicatricial pemphigoid patients). These studies have found that these patients are heterogeneous in that their autoantibodies target different regions of epidermal basement membrane as well as different keratinocyte antigens. These studies demonstrate that cicatricial pemphigoid is a disease phenotype rather than a single nosologic entity and that anti-epiligrin autoantibodies represent a specific immunologic marker for a unique subset of patients with these findings.

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

PROJECT NUMBER
Z01 CB 03638-25 D

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies of DNA Repair in Human Degenerative Diseases

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

PI: J. H. Robbins, M.D., Dermatology Branch, DCBDC, NCI
OTHER: V. A. Bohr, M.D., Senior Investigator, LMG, NIA
L. E. Nee, M.S.W., Senior Investigator, FSU, DIR, NINDS
K. S. Sanford, Ph.D., Senior Investigator, LCMB, DCE, NCI
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R. E. Tarone, Ph.D., Mathematical Statistician, BB, DCE, NCI

COOPERATING UNITS (if any)
Laboratory of Molecular Genetics, NIA; Laboratory of Cellular and Molecular Biology, DCE, NCI; Biostatistics Branch, DCCP, NCI; Family Studies Unit, DIR, NINDS; Laboratory of Neurosciences, NIA

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.8	1.3	1.5

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.)

Studies in this laboratory are designed to elucidate the role of DNA repair processes in carcinogenesis and in neurodegeneration. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP), who have defective DNA repair plus multiple cutaneous malignancies and premature aging of sun-exposed skin and of the nervous system. Cells from patients with primary neuronal and retinal degenerations are also being studied. These diseases include Cockayne syndrome, ataxia telangiectasia, Alzheimer disease, Parkinson disease, Huntington disease, and retinitis pigmentosa. These studies are designed to elucidate the pathogenesis of these disorders and to develop diagnostic tests. We assess the biological effectiveness of DNA repair by: 1) in vitro assays of cell survival after treatment of the cells with DNA-damaging agents; 2) analysis of chromosomal and chromatid aberrations in cells treated with DNA-damaging agents; and 3) determining DNA repair within defined genes as well as in the genome overall.

Project Description

Major Findings:

We have continued our collaboration with Drs. Katherine K. Sanford and Ram Parshad (Department of Pathology, Howard University College of Medicine) in the development of cytogenetic tests which shows very large differences in radiation-induced chromatid aberrations between normal and Alzheimer disease (AD) cells. We also collaborate with Ms. Linda E. Nee, who supplies us with the most recent clinical and pathological data concerning the patients whose cells we study from a large Canadian family with dominantly-inherited AD. We irradiated normal and AD skin fibroblasts in a 37°C 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 of the cell cycle, cells were exposed to the light for 5 hours and incubated an additional 15 hours, during the last hour of which Colcemid was added to arrest cells in metaphase. Thus, all metaphase cells examined had been in G_1 at the end of irradiation. Lesions not repaired in G_1 would persist into S phase. If these lesions inhibited DNA replication, they could result in gaps in newly synthesized DNA observable as chromatid aberrations in the succeeding metaphase. These gaps are normally mended during S phase by a daughter-strand, postreplication repair process which can be blocked by caffeine. Therefore, to retain the gaps present, half the cultures were exposed to caffeine from the end of irradiation through S and into G_2 phase.

In at least 14 of the 16 normal fibroblast lines studied caffeine led to no increase in the chromatid aberration frequency (CAF). However, cells from all 10 donors who were affected with AD in our Canadian family and whose fibroblasts were available to us had a marked increase in their CAF. It appears that the patients' cells, in contrast to the normal cells, had not repaired during G_1 all the fluorescent light-induced DNA damage. It should be noted also that the skin biopsies from three of these 10 donors were obtained years before the donors became symptomatic, documenting the presence of the DNA-repair defect in cells obtained from the donors prior to the onset of their neurologic symptoms.

We also studied lines from 11 asymptomatic sons and daughters of some of the ten symptomatic Canadian patients. Each of these offspring was at a 50% risk of having inherited the disease-producing AD gene in this family. Of these 11 donors, 8 had CAF values with caffeine well in the range given by patients with AD. We consider it highly likely that these 8 donors will eventually become symptomatic. Several of them were less than 32 years of age at the time of skin biopsy, suggesting that inheritance of the abnormal AD gene is manifested by our test in cells obtained at least two decades before the age of 52 years, when the disease typically produces symptoms in this family. Of the three at-risk donors who had low CAF values in the presence of caffeine, one died of cancer at the age of 49 years, and an autopsy of her brain showed no evidence to support a diagnosis of AD, indicating, therefore, that she had probably not inherited the disease-producing gene. We predict that the two other at-risk donors who also had low CAF values will not develop AD.

Our G_1 test should prove useful as a presymptomatic diagnostic test. In our Canadian AD family, it would make at-risk individuals informative in positional cloning studies to pinpoint the location on chromosome 14 of their defective AD gene. In other families, knowledge of which at-risk individuals harbor a defective AD gene should facilitate localization of the gene to the correct chromosome. Furthermore, presymptomatic diagnosis may be desired by some individuals at risk for familial AD to help guide them in personal decisions, including not only family planning but also participation in therapeutic intervention trials.

We are attempting to develop a cytogenetic test for AD using PHA-stimulated peripheral blood lymphocytes. Such a test would be quicker and easier to conduct than our current test using skin fibroblasts. We obtain blood from AD patients who are under the NINDS and NIA research protocols of Ms. Linda Nee and Dr. Mark Schapiro, respectively.

With Dr. V. A. Bohr, we have measured gene-specific DNA repair in a normal fibroblast cell line and in fibroblast lines from two patients with familial AD. Cells were treated with either ultraviolet radiation (UV) or with the chemotherapeutic alkylating agent, nitrogen mustard (HN2). DNA damage formation and repair were studied in the active dihydrofolate reductase (DHFR) gene for the main lesions introducted by each of these types of DNA-damaging agents. The gene-specific repair of UV-induced cyclobutane pyrimidine dimers in the human DHFR gene was 86% complete in the AD cells after 24 hours of repair incubation. This repair efficiency was similar to what we and others have found in normal human fibroblasts. After treatment of the AD cells with HN2, we found the frequency of HN2-induced lesions in the DHFR gene to be similar to the frequency in the transcriptionally inactive delta-globin gene. The gene-specific repair of HN2-induced lesions in the DHFR gene was completed with 8 to 24 hours in the normal fibroblast line and in the familial AD line, and the repair kinetics were similar for both cell lines. These results indicate that familial AD fibroblasts have normal gene-specific repair of both UV-induced and HN2-induced DNA damage. Our results, however, do not exclude the possibility that a minor, infrequent lesion induced by HN2 is not repaired normally in active genes in AD cells or that damage induced by other alkylating agents may be repaired deficiently in AD cells.

Publications:

None

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

PROJECT NUMBER
Z01 CB 03657-20 D

PERIOD COVERED
October 1, 1993 to September 30, 1994

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
A. Blauvelt, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI
C. Enk, Visiting Associate, Dermatology Branch, DCBDC, NCI
H. Asada, Visiting Fellow, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)

Dermatology Dept., USUHS, Bethesda
Experimental Immunology Branch, DCBDC, NCI

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	4.2	1

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 major area of study of this laboratory is the role of the epidermis as an immunological organ. We are continuing our studies of the very earliest events which occur during allergic contact dermatitis which we use as a paradigm for studying the skin immune system. Within 24 hours after exposure of epidermis to haptens, 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. There is an almost immediate upregulation of IL-1 β mRNA from Langerhans cells after hapten application, and later, an upregulation of IL-10 mRNA by keratinocytes. These cytokines exert functional effects on the skin, both in vivo and in vitro. Another area of intense interest is the nature of the precise epitopes generated after hapten-Langerhans cell interaction. Using Langerhans cells as antigen-presenting cells, we found that hapten-modified MHC class II binding peptides generate epitopes recognized by hapten-specific $CD4^+$ T cells and that precise positioning of hapten molecules on peptides fitting into the groove of the MHC class II molecules is required for optimal $CD4^+$ T-cell recognition. The findings provide insight into how haptens are recognized by T cells in contact sensitivity. Another major area of study is that of the role of epidermal Langerhans cells in HIV disease. We have just completed an extensive study of Langerhans cell and monocyte function in 21 HIV^+ and 21 HIV^- volunteers, including 3 monozygotic twin pairs discordant for HIV serology. Allostimulation by Langerhans cells and monocytes from HIV^+ individuals was impaired; however, results of identical twin studies indicate that protein-antigen presentation by these cells was intact. We conclude that immunologic dysregulation in early and intermediate stages of HIV disease does not reflect marked functional impairment of antigen-presenting cells, but may be secondary to T-cell dysfunction alone.

Project Description

Major 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 have shown that Langerhans cells express increased amounts of IL-1 β mRNA within 15 minutes after exposure to contact allergens. We have also found that IL-1 β mimics the effects of allergens on class II MHC antigen expression in their mobilization and migration to the regional lymph nodes. 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. We have continued our studies of IL-10 mRNA in human keratinocytes and the regulation of IL-10 gene transcription by ultraviolet B radiation. 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.

To better understand the nature of the precise epitopes generated after hapten-Langerhans cell interaction, we have begun assessing the ability of TNP-modified I-A binding peptides to activate hapten-specific $CD4^+$ T cells. Using Langerhans cells as antigen presenting cells, $I\text{-}A^k$ restricted TNP-specific $CD4^+$ T cells proliferated in the presence of the 10mer synthetic peptide hen egg lysozyme (HEL) 52-61 derivatized with TNP in position 56 (TNP-K56), and less so when TNP occupied positions 53 (TNP-K53) or 59 (TNP-K59). The requirement for direct interaction between TNP-peptides and MHC class II molecules for T cell recognition was also confirmed by the absence of response of haptenated peptides using BALB/c ($H\text{-}2^d$) $CD4^+$ T responder cells (from sensitized mice). Our results indicate that hapten-modified MHC class II binding peptides generate epitopes recognized by hapten-specific $CD4^+$ T cells and that precise positioning of hapten molecules is required for optimal $CD4^+$ T cell recognition. These finding provide insight into how haptens are recognized by T cells in contact sensitivity and should facilitate the study and design of specific therapies for the manipulation of hapten-specific $CD4^+$ T cell responses.

We are continuing our studies of the role of Langerhans cell in HIV. Although HIV reportedly infects Langerhans cells, the functional status of Langerhans cell in HIV infection is unknown. We studied Langerhans cell function and monocyte/macrophage function in 21 HIV-seropositive (HIV^+) and 21 HIV-seronegative (HIV^-) volunteers, including 3 monozygotic twin pairs discordant for HIV serology. Langerhans cells from HIV^+ individuals were quantitatively normal and expressed normal levels of HLA-DR. However, both Langerhans cells and monocytes from HIV^+ individuals stimulated allogeneic T cells less well compared to control APC. In addition, decreased protein antigen- and mitogen-induced T cell responsiveness was observed in HIV^+ individuals (particularly AIDS patients), using either autologous Langerhans cells or autologous monocytes as antigen presenting cells. Interestingly, Langerhans cells and monocytes isolated from the HIV^+ twins were able to present protein antigen normally to HIV-uninfected syngeneic T cells. Thus, although alloantigen presentation by Langerhans cells and monocytes from HIV^+ individuals was impaired, results of twin studies indicated that protein antigen presentation by these cells was intact. We conclude that immunologic dysregulation in early and intermediate stages of HIV disease does not reflect marked functional impairment of antigen presenting cells, but may be secondary to T cell dysfunction alone. (Time devoted to AIDS is 25%)

Publications:

Enk AH, Angeloni VL, Udey MC, and Katz SI. Inhibtion of Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance, J Immunol 1993;151:2390-2398.

Katz SI. The skin immune system: allergic contact dermatitis as a paradigm, J Dermatol 1993;20:593-603.

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Enk A, Katz SI. Heat stable antigen is an important costimulatory molecule on epidermal Langerhans cells, J Immunol 1994;152:3264-3270.

Sharrow S, Singer A, Katz SI. Quantitative differences in cell surface expression of class I major histocompatibility antigens on murine epidermal Langerhans cells, J Immunol 1994 (in press).

Book Chapters:

Katz SI. Dermatitis herpetiformis. In: Dermatology in General Medicine, Fitzpatrick, T.B. et al (Eds), McGraw Hill Co., New York, 4th Ed., 1993, 626-629.

Katz SI, Provost TT. Herpes gestationis. In: Dermatology in General Medicine, Fitzpatrick, T.B. et al (Eds), McGraw Hill Co., New York, 4th Ed., 1993, 626-629.

Katz SI. Erythema elevatum diutinum. In: Dermatology in General Medicine. Fitzpatrick, T.B. et al (Eds), McGraw Hill Co., New York, 4th Ed., 1993, 1167-1170.

Katz SI. Relapsing polychondritis. In: Dermatology in General Medicine. Fitzpatrick, T.B. et al (Eds), McGraw Hill Co., New York, 4th Ed., 1993, 2183-2185.

Katz SI. Dapsone. In: Dermatology in General Medicine. Fitzpatrick, T.B. et al (Eds), McGraw Hill Co., New York, 4th Ed., 1993, 2865-2868.

Book:

Burgdorf WHC, Katz SI, Editors: Dermatology: Progress and Perspectives. Proceedings of the 18th World Congress of Dermatology. Parthenon Press, 1993.

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

PROJECT NUMBER
Z01 CB 03659-20 D

PERIOD COVERED
October 1, 1993 to September 30, 1994

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: Robert B. Sollitto, M.D., Clinical Associate, Derm. Br., DCBDC, NCI
I. Tokar, Registered Nurse, Clinical Center Nursing
Maria Turner, M.D., Medical Officer, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)
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

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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

D

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 cornification. During the last decade over 300 patients studied have established the efficacy and characterized the toxicity of isotretinoin and etretinate for skin disorders. Patients requiring long-term therapy are monitored to characterize skeletal toxicity, an important side effect. After chronic etretinate therapy, a high rate of peripheral skeletal toxicity was first identified in these patients, and we have now discovered osteoporosis as a toxicity. In patients with high rates of skin cancer formation, we were first to demonstrate effective chemoprevention with oral isotretinoin and are further defining efficacy. In a phase I/II study of gamma interferon for basal cell carcinoma, all tumors treated became smaller, but few underwent complete regression. In patients with Darier's disease, we have identified a novel, common, cystic bone abnormality. Our collaboration with the Lab. of Skin Biology has grown. We were first to find linkage of the epidermolytic hyperkeratosis gene to the type II keratin gene cluster on chromosome 12q. We have now identified mutations in keratin 1 or 10 in 12 families. Based on our clinical studies, we created a new disease classification with 6 distinct clinical phenotypes; correlation with mutations will lead to a better understanding of the relationship between keratin structure and function. In a non-epidermolytic form of palmar/plantar keratoderma (Unna-Thost disease) we found complete linkage with the type II keratin region and discovered a mutation in the V1 end domain of the keratin 1 gene as the cause of this disease. Our fine mapping study of Darier's disease confirmed the location of the disease gene to chromosome 12q23-q24.1 and further narrows the area to a 5 cM interval. In lamellar ichthyosis we have found linkage to chromosome 14q11 and no recombination with the candidate gene transglutaminase 1.

Cooperating Units: (continued)

Nicholas Patronas, Radiology Department, Clinical Center
James Reynolds, M.D., Chief, Clinical Studies Sect., Nuclear Medicine, CC
Peter Steinert, Ph.D., Chief, Lab. Skin Biology, NIAMS

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 and the nevoid basal cell carcinoma syndrome. Additional patients are being screened. 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 and 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 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 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. In collaboration with James Reynolds from the Nuclear Medicine Department, we evaluated 24 patients with dual-photon and single photon absorptiometry to assess bone density. Bone mineral density was significantly decreased in the etretinate-treated group when evaluated against standardized data bases to control for age, sex and weight. Bone mineral density was also significantly decreased when compared to the group patients treated with isotretinoin. This important study is the first to document osteoporosis as a side effect of therapy with any synthetic retinoid.

Five patients have each had one basal cell carcinoma treated with 0.1 mg/M^2 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^2 and underwent complete histological regression but with moderate inflammation. The study is nearing completion with additional lesions being treated at 0.25mg/M^2.

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.

We have collaborated extensively with Sherri Bale, Peter Steinert, and John Compton from the Lab. of Skin Biology in an effort to better characterize clinical features and to map the genes for the disorders of cornification. We have focused on epidermolytic hyperkeratosis (EHK), recessive ichthyoses, and Darier's disease. We traveled to the midwestern U.S. to examine and obtain tissue samples from one family with 20 members affected with EHK 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 54 EHK patients and their unaffected relatives from 22 families. We have identified striking heterogeneity in clinical presentation between families with EHK. We have been able to distinguish 6 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 12 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. Interestingly, we have found the same arginine to histidine mutation in keratin 10 in different families with three distinct clinical phenotypes. This finding demonstrates that the specific keratin mutation is insufficient to determine the complete clinical phenotype. Further correlation of these clinical phenotypes and mutations will 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 consanguinous 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. From this group of recessive ichthyosis patients, we have selected a homogeneous subset with a clinically severe phenotype. Using this subset, we have mapped the locus for lamellar ichthyosis to a region on chromosome 14q11.

We have also established a collaboration with the Istituto Dermopatico Dell' Immacolata, Rome, Italy, to obtain families with ichthyosis vulgaris, Hailey-Hailey and Darier's disease (DD). Linkage analysis in 10 families with DD(34 affected) confirm recent reports mapping the DD gene to chromosome 12q23-24.1. Haplotype analysis of recombinant chromosomes have narrowed the location of the DD gene to a 5 cM interval.

Publications:

Goldstein AM, Bale SH, Peck GL, DiGiovanna JJ. Sun exposure and basal cell carcinomas in the nevoid basal cell carcinoma syndrome. J Am Acad Derm 29:34-41, 1993.

Bale SJ, Compton JG and DiGiovanna JJ. Epidermolytic hyperkeratosis. Seminars in Dermatology 12(3):202-209, 1993.

DiGiovanna JJ. Diversity of melanoma in humans, Clin Chem Enzym Comm 6:69-78, 1993.

Lippman SM, Ankrum M, DiGiovanna JJ. Oral retinoids in skin cancer. Cancer Bull 45:275-278, 1993.

DiGiovanna JJ, Bale SJ. Epidermolytic hyperkeratosis: applied molecular genetics. Progress in Dermatology 27(3):1-5, 1993; J Invest Derm 102:390-394, 1994.

Yang JM, Chipev CC, DiGiovanna JJ, Bale SH, Marekov LN, Steinert PM, Compton JG. Mutations in the H1 and 1A domains in the keratin 1 gene in epidermolytic hyperkeratosis. J Invest Derm 102:17-23, 1994.

Chipev CC, Yang JM, DiGiovanna JJ, Steinert PM, Compton JC, Bale SJ. Preferential sites in keratin 10 mutated in epidermolytic hyperkeratosis. Am J Hum Gen 54:179-190, 1994.

Goldstein AM, Pastakia B, DiGiovanna J, Poliak S, Santucci S, Kase R, Bale AE, Bale SJ. Clinical and genetic findings in two black families with the nevoid basal cell carcinoma syndrome (NBCC). Am J Med Genet 50:272-281, 1994.

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Lippman SM and DiGiovanna JJ: Retinoids in skin cancer. In Hong WK, Lotan R (Eds), Retinoids in Oncology ,Marcel Decker, New York, pp. 179-202, 1993.

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

PROJECT NUMBER
Z01 CB 03667-10 D

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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
Hema A. Sundaram, M.D., Clinical Associate, Dermatology Br., DCBDC, NCI
Peter J. Koch, Ph.D., Special Volunteer, Dermatology Br., DCBDC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6	5	1

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 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 have characterized, 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 1 (BPAG1) is in the plaque of the hemidesmosome. Molecular cloning of the full length coding sequence for BPAG1 indicates a similar domain structure to desmoplakin I, a desmosomal plaque protein. Current studies are aimed at determining the function of various subdomains of BPAG1 by transfecting cDNA that encode them into eukaryotic cells. The deduced amino acid sequence of PV antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules and is closely related to the PF antigen. PV antigen was localized ultrastructurally to the desmosome. Current studies are aimed at dissecting the functions of various extracellular and intracellular domains of PV antigen in cell adhesion and in binding to molecules (e.g. plakoglobin) in the desmosomal plaque.

Project Description

Major Findings:

Sequence analysis indicates that BP antigen 1 (BPAG1) is similar in structure and amino acid sequence to desmoplakin I, a desmosomal plaque protein, and to plectin, a keratin-binding protein.

cDNA cloning of PV antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules, most closely related to desmoglein I (dsg1). PV antigen is now also termed dsg3.

Rabbit antibodies raised against the extracellular domains of PV antigen bind to the external face of desmosomes in cultured keratinocytes.

PV patient antibodies affinity-purified on the extracellular domain of PV antigen and passively transferred to neonatal mice localize to separating desmosomes in their skin.

PV antigen (dsg3) and PF antigen (dsg1) are homologous at the level of amino acid sequence, molecular structure, and ultrastructural localization.

Transfection studies of mouse L cells indicate that the extracellular domain of PV antigen mediates weak homophilic adhesion.

Transfection studies with the cytoplasmic domain of PV antigen are being used to determine which domains are necessary for plakoglobin binding. Initial studies suggest that the ICS (intracytoplasmic cadherin-like seqment) is necessary.

Transfection studies with a chimeric E-cadherin/PV antigen molecule indicate that the cytoplasmic domain of PV antigen can confer adhesion ability on the extracellular domain of E-cadherin without binding cytoplasmic catenins, previously thought to be essential for adhesion function.

PV antigen does not share the biochemical properties that are characteristic of the classical cadherins (such as E-cadherin).

Publications:

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

PROJECT NUMBER
Z01 CB 03669-05 D

PERIOD COVERED
October 1, 1993-September 30, 1994

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,

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Other: Teresa A. Borkowski. M.D., Clinical Associate, DB, DCBDC, NCI
Kathyrn Schwarzenberger, M.D., Clinical Associate, DB, DCBDC, NCI
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.42	4.42	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.)

The laboratory is primarily involved in studies of leukocye-epithelial cell adhesion. We have determined that subpopulations of leukocytes that reside in mammalian epidermis (murine and human epidermal Langerhans cells (LC) and murine dendritic epidermal T cells) express the homophilic adhesion molecule E-cadherin. E-cadherin is also expressed by immature murine thymocytes in developing thymus as well as thymic epithelial cells in both developing and adult thymus. These results suggest that E-cadherin may play a general role in the adhesion of subpopulations of mammalian leukocytes to normal keratinizing epithelia in skin and other tissues. We have propagated dendritic cells from murine blood, bone marow and fetal skin and begun to use these cells to study the biology and biochemistry of E-cadherin expressed by leukocytes. Although dendritic cells expanded from blood, bone marrow and fetal skin all express E-cadherin, these cells do not adhere avidly to keratinocytes or other E-cadherin-expressing cells. Immunoprecipitation of E-cadherin and associated proteins from biosynthetically labelled fetal skin leukocyte extracts revealed proteins that co-migrate in SDS-PAGE with full length E-cadherin as well as α- and β-catenin (proteins that couple cadherins to the cytoskeleton). Thus, failure of these cells to exhibit high affinity E-cadherin-mediated binding does not result from an obvious abnormality in E-cadherin or a deficiency in cadherin-associated proteins known to be required for full expression of cadherin activity. Experiments designed to determine if the affinity of E-cadherin expressed by Langerhans (or related) cells is regulated via tyrosine phosphorylation of E-cadherin or β-catenin, or by *wnt* proto-oncogene products within skin are in progress. We are also interested in elucidating the role that TGFβ1 plays in Langerhans cell development. We have determined that TGFβ1 augments the outgrowth of Langerhans cell-like cells from fetal skin and selectively induces expression of a novel Langerhans and dendritic cell differentiation antigen by dendritic cells expanded from bone marrow. We have also evaluated TGFβ1 knockout mice and have determined that they are essentially devoid of epidermal Langerhans cells. Additional experiments will be required to determine if other tissues contain normal complements of dendritic cells, and to determine if the deficiency of Langerhans cells results from an absence of bone marrow-derived precursors or reflects a defect in the cutaneous microenvironment. Whether or not there is any interaction between TGFβ1 production and E-cadherin expression or affinity also remains to be determined.

Project Description

Major Findings

Last year we reported that murine epidermal Langerhans cells expressed the homophilic adhesion molecule E-cadherin, and demonstrated that E-cadherin mediated adhesion of Langerhans cells to keratinocytes in vitro. This represented the first report of expression of any member of the cadherin supergene family of adhesion molecules by leukocytes, and has provided for new insights into mechanisms that mediate interactions between leukocytes and epithelial cells. In the interim, we have determined that expression of E-cadherin appears to be a general feature of Langherhans cells, in as much as human as well as mouse epidermal Langerhans cells express E-cadherin. We have also completed a survey of murine lymphoid dendritic cells and determined that Langerhans cells are unique among dendritic cells in their ability to express high levels of E-cadherin. Dendritic cells from spleen and gut-associated lymph nodes failed to express E-cadherin. Low levels of E-cadherin are expressed by dendritic cells isolated from skin-associated lymph nodes, however, consistent with the proposed lineage relationship between these cells and epidermal Langerhans cells. A subpopulation of thymic dendritic cells may also express E-cadherin.

In an attempt to develop a model system that will permit studies of mechanisms that regulate the level of expression and affinity of E-cadherin molecules on the surfaces of Langerhans cells or related cells, we have propagated dendritic cells from murine blood, bone marrow and skin in vitro. Cells grown from blood and bone marrow in media supplemented with GM-CSF express differentiation antigens typical of several kinds of dendritic cells, and also express E-cadherin. Based on the surface antigens that they express, dendritic cells obtained from cultures of blood and bone marrow are not identical to any single population of tissue dendritic cells including epidermal Langerhans cells, however. In addition, although they express E-cadherin, these dendritic cells do not adhere avidly to kerationocytes or other cells that express E-cadherin. Thus, it is not obvious that dendritic cells propagated from blood or bone are suitable for future studies. We have also expanded leukocytes from fetal skin in GM-CSF-supplemented media with the expectation that these cells might resemble Langerhams cells more closely than cells obtained from blood or bone marrow. We have found that leukocytes propagated from fetal skin exhibit a uniform surface phenotype that is identical to that of epidermal Langerhans cells, with the important exception that most fetal skin leukocytes do not express class II MHC antigens. However, co-culturing fetal skin leukocytes in GM-CSF and IFN-γ results in expression of cell surface class II MHC antigens, and in acquisition of enhanced accessory cell activity. To affirm that these cells represent an appropriate model system, it is important to determine if these cells can give rise to Langerhans cells in vivo. Whether or not leukocytes expanded from fetal skin can give rise to Langerhans cells will be tested by transferring them into MHC-disparate immunodeficient mice and assaying their ability to take up residence in skin, express class II MHC antigens and assume the approriate morphology. Preliminary results suggest that leukocytes expanded from C57BL/6 fetal skin have the capacity to become Langerhans cell-like after engaftment onto BALB/c nu/nu mice in combination with BALB/c keratinocytes and fibroblasts. The ability of leukocytes propagated from fetal skin to evolve into Langerhans cells after subcutaneous and systemic admistration will also be tested.

We have begun to utilize leukocytes from fetal skin to study the biology and biochemistry of cadherins expressed by leukocytes. In primary cultures of fetal skin, Langerhans cell-like cells first appear in clusters associated with stroma. At this stage, these leukocytes adhere very tightly to each other, suggesting that cadherins are active. Cell lines derived from primary cultures of fetal skin cells exhibit a stable Langerhans cell-like phenotype, and have been maintained in culture for more than four months in the absence of stromal cells. Although they continue to express E-cadherin, these cells lose the ability to adhere to each other, suggesting that the cadherins that they express are not active. Immunoprecipitation of biosynthetically-labelled fetal skin leukocyte lysates with anti-E-cadherin mAb reveals proteins that co-migrate in SDS-PAGE with full-length E-cadherin as well as α- and β-catenin. Confirmation of the identity of these latter bands with epithelial cell-derived α- and β-catenin awaits the results of immunoblotting studies carried out with polyclonal anti-catenin

Abs. However, it seems unlikely that the failure of fetal skin-derived leukocytes to exhibit high affinity E-cadherin mediated binding reflects an abnormality in E-cadherin structure or a deficiency in the proteins that couple cadherins to cytoskeletal elements. Recent data from other laboratories indicates that cadherin affinity can be actively regulated via post-translational mechanisims; mechanisms that may be operative in Langerhans cells and related cells.

E-cadherin and β-catenin are tyrosine phosphorylated in previously transformed cells after transfection with *src*-family tyrosine kinase-encoding oncogenes. Introduction of these oncogenes into transformed cells also results in decreased cell cohesiveness and an increased tendency to invade soft agar. Since cell surface levels of E-cadherin do not decrease and mutated oncogenes encoding inactivated tyrosine kinases do not affect adhesive or invasive potential, increased tyrosine phosphorylation of cadherin and/or β-catenin is apparently associated with decreased cadherin affinity. Although cadherin function has not yet been shown to be regulated by differential phosphorylation of E-cadherin or β-catenin in normal cells, we speculate that this may occur in Langerhans cells after activation by antigen and prior to emigration of Langerhans cells from epidermis. Experiments designed to determine if these proteins are tyrosine phosphorylated in fetal skin leukocytes, and to determine if the affinity of E-cadherin expressed by fetal skin leukocytes can be modulated by agonists and antagonists of tyrosine phosphorylation are in progress.

Cadherin affinity can also by regulated by products of the proto-oncogene *wnt-1* and the homologous *drosophila* gene *wingless*. One of the effects of these pleotropic genes is increased affinity of β-catenin (or the *armadillo* gene product in *drosophila*) for E-cadherin (or *drosophila* cadherin), resulting in increased cadherin affinity. Because *wnt* and *wingless* gene products are secreted proteins that adhere tightly to cells and matrix in the vicinity of their production, these genes are expected to have only short range effects. We hypothesize that *wnt* family members are expressed in skin and *wnt* gene products may modulate cadherin affinity on Langerhans cells and related cells. If this is the case, it is possible that *wnt* genes could promote localization of Langerhans cells in epidermis by activating Langerhans cell cadherin molecules within the cutaneous microenvironment. In preliminary experiments, we have detected *wnt*-related mRNA in RNA derived from skin, suspensions of epidermal cells (mostly keratinocytes) and mixed cultures of fetal skin leukocytes and stromal cells using degenerate primers and RT-PCR methodology. The cell source, exact nature and potential role of these *wnt*-related gene products in Langerhans cell biology will be addressed in future experiments.

We are also interested in the role that cadherins may play in interactions between leukocytes and epithelia in the thymus. We have determined that most murine fetal day (FD) 14 thymocytes and esssentially all FD16 thymocytes express E-cadherin. Thereafter, the proportion of thymocytes expressing E-cadherin and the level of E-cadherin expressed by individual thymocytes decreases with increasing gestational age such that very few adult thymocytes express E-cadherin. E-cadherin is also expressed by thymic epitheilial cells throughout fetal and adult life. The E-cadherin expressed by fetal thymocytes is synthesized by the thymocytes and is not adsorbed from epithelial cells, however. Multicolor flow cytometry carried with anti-E-cadherin mAb and mAb directed against various thymocyte differentation antigens indicates that maximal levels of E-cadherin are expressed by the most immature thymocytes present at each gestational age. These observations suggest that E-cadherin is involved in important interactions that occur between thymocytes and stromal elements early in the course of thymocyte differentiation. The potential functional importance of E-cadherin-mediated interactions between thymocytes and thymic epithelia cells will be studied by examining the impact of anti-E-cadherin mAb on reaggregation of fetal thymus fragments, and proliferation and differentiation of thymocytes in fetal thymic explants in vitro. It will also be interest to determine if E-cadherin plays a role in the localization of prothymocytes in the epithelial thymic rudiment by assessing the ability of anti-E-cadherin mAb to inhibit repopulation of thymocyte-depleted fetal thymic lobes by fetal liver-derived precursors in vitro.

The remaining area of major interest in the laboratory involves elucidating the role that TGFβ1 plays in Langerhans cell ontogeny. We have observed that TGFβ1 markedly enhances the outgrowth of cells resembling

Langerhans cells from fetal skin, and also induces expression of a novel Langerhans cell and dendritic cell differentiation antigen by dendritic cells propagated from bone marrow. These observations provided the impetus to evaulate Langerhans cells in the skin of TGFβ1 knockout mice. We have determined that the skin of TGFβ1 knockout mice is essentally devoid of epidermal Langerhans cells. Experiments designed to determine if the defect is restricted to only Langerhans cells (as compared with other dendritic cells) and to determine if the deficiency is reflective of an abnormally low number of bone marrow-derived precursors or a defect in the cutaneous microenvironment are in progress.

We believe that the discovery that epidermal Langerhans cells express E-cadherin provides us with an important clue to understanding mechanisms that mediate Langerhans cell-keratinocyte adhesion and Langerhans cell localization in epidermis. Based on results obtained in the last year, we have the overiding impression that E-cadherin affinity is actively regulated by local microenviromental influences within skin. We anticipate that the next several years will represent an exciting time during which the cytokines, proto-oncogenes and other influences that regulate cadherin function within skin will become better characterized, and mechanisms by which they regulate cadherin function will be precisely determined.

Publications

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Enk AH, Angeloni VL, Udey MC and Katz SI. Inhibition of Langerhans cell antigen-presenting fucntion by IL-10. A role for Il-10 in the induction of tolerance. J. Immunol. 151: 2390-2398, 1993.

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Amagai M, Karpati S, Klaus-Kovtun V, Udey MC and Stanley JR. The extracellular domain of pemphigus vulgaris antigen (desmoglein 3) mediates weak homophilic adhesion. J. Invest. Dermatol. 102: 402-408, 1994.

Plott RT, Amagai M, Udey MC and Stanley JR. Pemphigus vulgaris antigen lacks biochemical properties characteristic of classicla cadherins. J. Invest. Dermatol. (In press).

Lee MG, Sharrow SO, Farr AG, Singer A, and Udey MC. Expression of the homotypic adhesion molecule E-cadherin by immature thymocytes and thymic epithelial cells. J. Immunol. (In press).

Foss FM, Borkowski TA, Gilliom M, Steler-Stevenson MA, Jaffe ES, Figg WD, Tompkins A, Bastian A, Nylen P, Woodworth TA, Udey MC and Sausville EA. Chimeric fusion protein toxin $DAB_{486}IL\text{-}2$ in advanced mycosis fungoides and Sezary syndrome: Correlation of activity and interleukin-2 receptor expression in a phase II study. Blood (In press).

Journal Supplements:

Borkowski TA and Udey MCl Langerhans cell biology: A progress report. J. Invest. Dermatol. (In press).

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

PROJECT NUMBER
Z01 CB 03670-02 D

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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Other: Ulrich Hengge M.D., Visiting Fellow, Derm/DCBDC/NCI,
Ruth Ann Foster, General Fellow, Derm/DCBDC/NCI,
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TOTAL STAFF YEARS: 4.17	PROFESSIONAL: 4.17	OTHER:

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☐ (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 goal of this laboratory is to develop methods for stable introduction and expression of genes in miniature swine keratinocytes for gene therapy purposes. The in vivo approach directly introduces the gene into epidermis while in our ex vivo approach, we first isolate keratinocytes or skin organ cultures, insert the desired gene while in tissue culture, and graft the genetically modified keratinocytes back onto the donor. Selection for keratinocyte stem cells or early precursors which have desired gene integrated into their genome is an important feature of our approach. For the purpose of both in vivo and ex vivo selection, the indicator gene ß-galactosidase (ßGal) has been linked to selectable marker genes which provide resistance to toxic agents such as G418 (Geneticin), Hygromycin, and the multi-drug resistant (MDR) gene agents colchicine and vincristine. We have developed a novel in vivo approach for directly introducing and expressing the ßGal indicator gene in epidermis by injecting naked plasmid DNA into the dermis underlying the epidermis. The naked DNA traverses the dermal-epidermal junction and is absorbed and expressed by keratinocytes. Colorimetric histochemical assays reveal uptake and expression of ßGal plasmid DNA in keratinocytes throughout the epidermis at all stages of differentiation. A quantitative assay for ßGal enzyme expression in epidermis shows that a dose response relationship exists for increasing concentrations of injected naked plasmid DNA. The presence of ßGal enzyme can be demonstrated for 3 weeks following injection, but at later time points the stable enzyme is only present in the outer differentiated keratinocyte layers. Analysis of ßGal RNA expression in epidermis shows expression for only 1 week suggesting that the stable ßGal enzyme can persist in the epidermis even though no longer actively expressed. Topical application of the selecting agents G418 (Geneticin) and Hygromycin was not successful for in vivo selection of keratinocytes with integrated plasmid DNA. Colchicine and vincristine appear to be superior for in vivo selection following topical application. Ex vivo approaches utilizing selection have also been devised. The MDR gene has been transduced into primary keratinocytes using defective retroviral vectors and keratinocytes expressing the MDR gene have been selected in vitro by using colchicine and vincristine. We are now attempting to successfully graft these keratinocytes back onto the donor miniature swine. Ex vivo organ cultures of skin, which have re-epithelializing keratinocytes, have also been both transfected and transduced with liposome-coated plasmid DNA and combinations of retroviruses respectively. These organ cultures can be selected and grafted back onto the donor with subsequent topical selection with colchicine or vincristine. The main advantage of the ex vivo approach is that a much higher percentage of the keratinoctyes have the desired gene stably integrated into the genome, ensuring persistent expression.

Project Description

Major Findings and Proposed Studies

The main focus of this laboratory is to develop novel methods to stably introduce and express candidate genes into keratinocytes, which constitute more than 90% of mammalian epidermis, for the purpose of in vivo gene therapy. Keratinocytes could be used to express protein products such as growth factors, cytokines, or enzymes for systemic delivery, or could express normal keratinocyte proteins which could treat specific skin diseases caused by a genetic defect of an endogenous keratinocyte protein. Miniature swine are being used for these studies because their epidermis is morphologically similar to human epidermis and has been a successful model for wound healing in human skin.

Successful keratinocyte gene therapy requires the gene be present in stem cells or very early precursors in order to maintain and express the gene over time in a high percentage of keratinocytes. Our in vivo approach of keratinocyte gene therapy directly introduces the gene into the epidermis while the ex vivo approach first isolates keratinocytes from the epidermis, inserts the desired gene in tissue culture, and grafts the genetically modified keratinocytes back onto the donor. To overcome the problem of gene persistence in the epidermis, we are developing methods to not only select in tissue culture, but continue to select in vivo when the genetically modified keratinocytes are part of the epidermis by continuous topical application of the selective agent. The concept of selection in this context is analogous to tissue culture selection where reagents such as G418 (Geneticin) are used to select for cells which have been stably transfected with plasmids that contain both a resistance gene and the desired candidate gene.

A novel in vivo approach for introducing and expressing genes in epidermal keratinocytes is to inject naked plasmid DNA directly into the subepidermal dermis (superficial dermis). After injection, plasmid DNA containing the ß-galactosidase (ßGal) indicator gene is surprisingly taken up and expressed by keratinocytes at all levels of the epidermis and at all states of differentiation, implying that the naked DNA rapidly traverses the dermal-epidermal junction and is then absorbed by keratinocytes. The expression of indicator genes, under the regulatory control of different viral promoters such as CMV, RSV, and SV40, is easily demonstrated histologically using colorimetric and immunofluorescent assays. In order to better quantitate the amount of ßGal produced by keratinocytes, we devised a quantitative assay for ßGal expression in tissue using a chemiluminescent substrate which is activated by ßGal and can be photometrically quantitated. Dose response curves showed that increasing doses of injected naked plasmid DNA produced increasing amounts of ßGal with a linear response up to 25 μg of injected plasmid DNA. The absolute amount of ßGal produced by the epidermis in an injected area was approximately 0.05% of the total cellular protein extract which is comparable to the amount produced by muscle following injection of naked DNA. Previously, muscle has been reported as the only tissue to express naked DNA in vivo. To determine how long the ßGal DNA persists and is expressed following injection, one can assay for the presence of ßGal enzyme and ßGal mRNA expression. Colorimetric histochemical analysis shows that ßGal enzyme is present in the epidermis for at least three weeks, but after 1 week, most of the ßGal enzyme is located in keratinocytes of the outer epidermal layers and is no longer found in the basal layers. RNA expression of the ßGal gene, as determined by reverse-transcriptase PCR (RT-PCR), declines after about one week. Southern analysis of genomic DNA and Hirt extracts are currently being performed to determine both the persistence and integration status of the absorbed plasmid DNA, but initial results suggest that no detectable plasmid has integrated into the genomic DNA of keratinocytes. These results are consistent with transient expression of

the ßGal gene following uptake by the epidermis, yielding a stable ßGal enzyme which persists until it is eventually lost from the epidermis.

Topical in vivo selection will be required to increase the number of keratinocytes persistently maintaining and expressing the desired gene by selecting for those epidermal keratinocytes containing both the ßGal indicator gene and an integrated selectable marker gene. Reagents such as G418 and Hygromycin, proved to be unacceptable for topical in vivo selection in miniature swine. G418 (Geneticin) induces allergic reactions in miniature swine skin and the concomitant use of steroids only delayed the allergic reaction. Hygromycin was capable of inducing cell death in epidermal keratinocytes, but was ultimately not acceptable for long term selection for stably integrated cells because the amount of cell death was very difficult to titrate and the cell death invariably led to extensive inflammation which would presumably destroy cells whether or not they contained the resistance genes. Another class of resistance genes that appear to be superior for our purposes are the multi-drug resistance genes (MDR) which confer resistance to a diverse group of drugs including mitotic blockers such as colchicine and vincristine. These agents appear to be superior when applied topically to the epidermis because they exert their selective effects primarily on the dividing basal layer and do not cause the widespread cell death and inflammation found with the previous selective agents. Additionally, these agents have titratable effects following topical application. We would like to inject either retroviruses or plasmids that contain both the MDR gene and an indicator gene followed by topical selection to determine if we can select for long term persistence and expression.

Methods based on the ex vivo approach are most likely to lead to long-term persistence and expression of genes in keratinocytes. The ex vivo approach has the advantage of allowing selection both in vitro after the gene has been introduced into the keratinocytes and in vivo topically, after the genetically modified keratinocytes have been grafted back onto the donor. Previously we have described how we developed the necessary culture conditions for growing miniature swine keratinocytes. MDR-containing retroviruses have been used to stably introduce the MDR gene into primary keratinocytes in tissue culture and we have been able to select these primary keratinocytes into pure populations which express high levels of MDR as evidenced by their resistance to agents such as vincristine and colchicine and confirmed by FACS analysis. Our current goal is to graft these genetically modified keratinocytes back onto a donor pig and continue topical selection in vivo. However, we have had difficulties grafting these primary miniature swine keratinocytes back onto the donor pigs using either artificial dermal equivalents or de-epidermized miniature swine dermis. We currently are trying to optimize conditions for miniature swine keratinocytes.

Ideally, both the candidate gene (ßGal) and the MDR gene would be present in the same transducing retrovirus so that selection would yield keratinocytes expressing both MDR and ßGal. Since there are limits to the size and number of candidate genes which can be inserted into these defective retroviral vectors, this approach may not always be feasible. We have been working on techniques to transduce primary keratinocytes with two different defective retroviral vectors at the same time, one carrying the MDR gene, the other carrying the ßGal candidate gene. This co-transduction approach appears to be effective with immortalized NIH-3T3 cell lines and needs to be adapted and optimized in primary keratinocytes.

Another ex vivo approach we have been pursuing that would circumvent the difficulties we have had in grafting primary keratinocytes is to insert the genes into the epidermal keratinocytes of a skin (epidermis-dermis) organ culture. In brief, we have created an in vitro model of re-epithelialization in which keratinocytes proliferate and migrate across an bare dermis which has had its keratinocytes removed by gentle trypsinization. The new keratinocytes are derived from keratinocytes remaining in the hair follicle and are targets for

gene insertion as they proliferate and differentiate on the dermis while in tissue culture. To date, we have been able to insert both liposome-coated plasmid DNA at relatively low levels, and also transduce these cells with retroviral vectors containing ßGal genes. Our goal is to use this re-epithelialization model for insertion of both candidate and selectable genes and select this organ culture in vitro and continue selection after grafting the organ culture back onto the donor. Since the organ culture is similar to real skin, it is easier to graft than primary keratinocytes. The main advantage of the ex vivo approaches over the above in vivo approach is that a much higher percentage of the keratinocytes have the desired gene stably integrated into the genome which will ensure persistent expression.

Many different constructs have been fabricated to pursue the above goals. A key issue for long-term expression is to have the appropriate promoter to drive expression of the candidate gene in the target tissue. Towards that goal we have cloned a keratinocyte-specific regulatory region (2kb of 5'-flanking sequence of the K14 keratin gene) which should be superior to many of the viral promoters we have used at expressing at high level for long periods of time. Other keratinocyte-specific promoters not available may be cloned in the future.

Publications

Vogel JC. Keratinocyte gene therapy, Arch Dermatol 1993;129:1478-1483.

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

PROJECT NUMBER
Z01 CB 03671-01 D

PERIOD COVERED
October 1, 1993 to September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mechanisms of keratinocyte adhesion to epidermal basement membrane

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

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Gudula Kirtschig, M.D., Special Volunteer, Dermatology Branch, DCBDC, NCI
Luca Borradori, M.D., Special Volunteer, Dermatology Branch, DCBDC, NCI

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5	4	1

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☐ (a1) Minors
☐ (a2) Interviews

A

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

The specific goal of my laboratory is to define molecules that promote adhesion of human keratinocytes to epidermal basement membrane. This fundamental issue is addressed in both clinical and basic investigative studies. In regard to the former, my laboratory studies patients with acquired autoimmune and inherited subepithelial bullous diseases. By defining and characterizing molecules targeted by autoantibodies in patients with acquired autoimmune bullous diseases, knowledge is gained about important structural proteins in skin as well as disease pathophysiology. My laboratory has a specific interest in bullous diseases in which patients have autoantibodies directed against bullous pemphigoid antigens 1 and 2, laminin 5 (also called kalinin or nicein), type VII collagen, or other yet to be defined molecules. Similarly, by identifying proteins that are defectively expressed in the skin of patients with inherited subepithelial bullous diseases, my laboratory hopes to develop an understanding about the key role that such proteins play in epidermal homeostasis. My laboratory has a specific interest in various forms of junctional epidermolysis bullosa, inherited subepithelial bullous diseases suspected to be associated with mutations in proteins that promote adhesion of basal keratinocytes to epidermal basement membrane. The identification of defective structural proteins in the skin of patients with such inherited bullous diseases will facilitate the identification of mutations in genes encoding these proteins, the prenatal diagnosis of these disorders, and their treatment (as technology to introduce exogenous genes into human keratinocytes is developed). Building on findings in these clinical investigations, my laboratory isolates native and recombinant forms of keratinocyte adhesion molecules to investigate their interactions with each other as well as with proteins in extracellular matrix. The goal of these in vitro studies is to further our understanding of epidermal morphogenesis, homeostasis, and differentiation.

Major Findings

1. My laboratory has identified patients with an acquired subepithelial bullous disease who have IgG autoantibodies directed against a complex of disulfide-linked human keratinocyte polypeptides of 170, 145, 125, and 95 kD. Comparative immunoprecipitation and preclearance studies have shown that this complex of polypeptides comigrates in sodium dodecyl sulfate polyacrylamide gel electrophoresis with epiligrin and laminin 5 - closely related adhesion proteins in skin that promote attachment of epidermis to epidermal basement membrane (see below). In addition, we (and others) have shown that these patients' autoantibodies as well as monoclonal antibodies directed against epiligrin or laminin 5 do not bind the skin of individuals with Herlitz's disease (lethal junctional epidermolysis bullosa [JEB]), an inherited subepithelial bullous disease characterized by separation of epidermis from epidermal basement membrane. These studies demonstrate that epiligrin is abnormal in both an acquired autoimmune as well as in an inherited subepithelial bullous disease.

2. We have extended our studies of patients with anti-epiligrin autoantibodies by characterizing additional patients with similar clinical and immunopathological findings (i.e., cicatricial pemphigoid patients) Our studies have found that these patients are heterogeneous in that their autoantibodies target different regions of epidermal basement membrane as well as different keratinocyte antigens. These studies demonstrate that cicatricial pemphigoid is a disease phenotype rather than a single nosologic entity and that anti-epiligrin autoantibodies represent a specific immunologic marker for a unique subset of patients with these findings.

3. Recent studies in other laboratories have suggested that epiligrin in human keratinocyte extracellular matrix represents a complex of several different proteins one of which is laminin 5 (a laminin isoform also called nicein or kalinin). To further specify the reactivity of autoantibodies in our patients with the above described unique form of cicatricial pemphigoid, we have tested their sera for evidence of reactivity against laminin 5 partially purified from SCC-25 media or human keratinocyte extracellular matrix. These studies have found that autoantibodies from all patients with this form of cicatricial pemphigoid bind the A chain of laminin 5 by immunoblot. Moreover, it has been possible to affinity purify patient autoantibodies against the A chain of laminin 5 immobilized on nitrocellulose and show that these antibodies bind the dermal side of 1 M NaCl split skin (in the same manner as autoantibodies in patient sera). Because the A chain of this heterotrimer is felt to be important in mediating keratinocyte adherence, our findings suggest that patient autoantibodies may impair the function of this adhesion protein in skin in vivo and play a direct role in disease pathogenesis.

4. To extend our studies of patients with inherited subepithelial bullous diseases, we have assessed expression of laminin 5 and related adhesion molecules in the epidermal basement membrane of patients with generalized atrophic benign JEB. Patients with this rare disorder have extensive skin and mucosal blisters that heal with atrophy and scarring; other manifestations include alopecia, nail dystrophy, and large acquired melanocytic nevi at sites of prior blisters. In contrast to the aforementioned studies of patients with lethal JEB, we

have found that multiple antibodies directed against laminin 5 bind these patients' epidermal basement membrane normally. Moreover, bullous pemphigoid antigen 1 as well as integrin receptors a_6b_4 and a_3b_1 are also expressed normally in the skin of these patients. However, we have recently found that two monoclonal antibodies directed against the extracellular domain of type XVII collagen, a transmembrane component of hemidesmosomes in basal keratinocytes, show diminished reactivity against the skin of four siblings with generalized atrophic benign JEB. These findings identify a defective adhesion molecule in patients with a genodermatosis characterized by blister formation at the exact location where this protein is located in human epidermal basement membrane. The gene encoding type XVII collagen is suspected as a leading candidate for mutations responsible for this disease.

Future Directions

1. Recent studies in other laboratories have shown that a monoclonal antibody (BM165) directed against the A chain of laminin 5 impairs keratinocyte adhesion to extracellular matrix in vitro. Because autoantibodies from our unique subset of patients with cicatricial pemphigoid target the same polypeptide, these findings raise the possibility that our patients' autoantibodies may be directly responsible for blister formation in their lesional skin. A specific aim of future studies is to address this hypothesis using patient autoantibodies as well as high titer polyclonal antibodies from rabbits immunized with laminin 5 to determine if these antibodies will:

a. impair human keratinocyte adhesion to extracellular matrix, plastic, or purified matrix proteins (specifically, laminin, type IV collagen, or fibronectin).

b. promote human keratinocyte dysadhesion from extracellular matrix, plastic, or purified matrix proteins.

c. induce separation of epidermis from epidermal basement membrane in vitro.

d. cause blister formation in vivo following passive transfer to neonatal BALB/c mice.

2. Type XVII collagen (also called bullous pemphigoid antigen 2) is a transmembrane molecule found in the hemidesmosomes of basal keratinocytes that is thought to play a key role in several bullous diseases. Specifically, prior studies have conclusively shown that autoantibodies from patients with the subepidermal blistering diseases bullous pemphigoid and herpes gestationis target this molecule. It has also recently been suggested that autoantibodies from certain patients with cicatricial pemphigoid bind this molecule. Moreover, as mentioned above, we have recently found that this protein is defectively expressed in the epidermal basement membrane of patients with generalized atrophic benign JEB. For these reasons, we are currently isolating cDNAs corresponding to various portions of this molecule. Our specific aim is to use these cDNAs to express recombinant forms of this molecule in bacteria and baculovirus. These recombinants will be used to:

a. epitope map the reactivity of autoantibodies from patients with bullous pemphigoid, herpes gestationis, and cicatricial pemphigoid against type XVII collagen.

b. assess the interactions of this molecule with other matrix proteins in human keratinocyte extracts and culture media. (Note: A leading candidate to bind type XVII collagen is laminin 5 since the former is located in basal keratoinocyte hemidesmosomes and positioned immediately above the latter in anchoring filaments. Moreover, we now suspect that mutations in either moiety will produce a clinical disease (i.e., a form of JEB) with a similar phenotype.)

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SUMMARY REPORT
EXPERIMENTAL IMMUNOLOGY BRANCH
October 1993 - September 1994

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 T cell receptor positive (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. Using mice which coexpressed the scid defect and transgenes encoding clonotypic TCR chains, it was found that maturation and organization of medullary thymic epithelium required thymocytes which expressed TCRαß heterodimers. These studies are important to our understanding of T cell differentiation and emphasize the critical nature of reciprocal interactions between thymocytes and thymic stroma in T cell development.

The nature of TCR assembly and expression were studied by Dr. A. Singer's laboratory using genetically altered mice transgenic for two different TCRα and two different TCRß proteins (9273). It was found that while each TCRα paired with each TCRß chain, each TCR complex contained only a single TCRα and a single TCRß protein. It was also found that individual T cells expressed twice as many CD3ε proteins as TCRß proteins. These findings demonstrate that each TCR/CD3 complex expressed on the surface of thymocytes contains precisely one TCRα, one TCRß and two CD3ε chains.

In other studies, the role of the TCR component zeta chain was studied by Dr. A. Singer's laboratory using genetically altered mice (9273). It was found that zeta performs a previously unappreciated role in quantitatively promoting the generation and/or expansion of $CD4^+CD8^+$ thymocytes and is critical for the generation of CD4 or CD8 single positive mature T cells. It was also found that overexpression of full length zeta chain caused premature termination of expression of recombination activating genes, prevented productive rearrangement of the TCRα and TCRß genes and blocked entry of thymocytes into the CD4/CD8 developmental pathway. These results suggest that an early signaling pathway which regulates TCR gene recombination exists in precursor thymocytes and is differentially responsive to individual members of the zeta protein family.

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 $CD4^-CD8^{lo}$ phenotype into $CD4^+CD8^+$ double positive thymocytes (9273). 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 $CD4^-CD8^{lo}$ precursor thymocytes into double positive thymocytes, and this progression is specifically regulated by interaction with cortical thymic epithelial cells (TEC). The regulatory ligands expressed by cortical TEC were identified as transforming growth factor ß1 (TGF-ß1) and TGF-ß2. These studies identify a novel regulatory mechanism that acts on developing precursor thymocytes independently of TCR, and that is mediated by cortical TEC.

The contribution of non-TCR mediated signals to thymocyte negative selection was also studied by Dr. A. Singer's laboratory by analysis of requirements for the in vitro induction of apoptosis (9273). It was found that TCR engagement alone does not efficiently induce apoptosis of double positive thymocytes. However, signals generated by simultaneous engagement of TCR and the costimulatory molecule CD28 delivered a potent apoptotic signal, demonstrating that both TCR and costimulatory signals are necessary to induce thymocyte apoptosis. These results provide a molecular basis for differences among cell types in their ability to mediate negative selection of developing thymocytes.

Negative selection, the process 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) Vß products were detected, indicating that maintenance of tolerance to a variety of self determinants results in substantial deletions in the available TCR Vß repertoire. The self determinants that function as ligands for Vß-specific T cell deletions were the products of non-MHC-encoded genes in association with MHC gene products. Mapping of the non-MHC-encoded genes contributing to Vß-specific deleting ligands has in each case identified an endogenous mouse mammary tumor (MMTV) provirus associated with deletion.

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 Vß2 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 Vß14 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.

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 $CD4^+$ T cells expressing Vß5, followed later in the course of infection by widespread immune deficiency in all T cells. These findings indicate the effect of Vß-specific, superantigen-like stimulation in vivo and in vitro in response to MAIDS retrovirus.

Although Vß-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 α and ß chain components (9265). Analysis of the expression of specific TCR Vα/Vß pairs has indicated that Vα/Vß pairing is non-random, and that strain-specific differences exist in patterns of Vα/Vß expression, providing a new approach to the study of repertoire selection. Additional evidence for the importance of Vα/Vß combinatorial specificity was observed in the response to the endogenous superantigen Mls^a (mtv-7). When Mls^a-specific T cells were selected by in vitro stimulation, it was found that Vα expression, in addition to the dominant influence of Vß expression, plays a role in T cell specificity for endogenous mtv superantigen.

Dr. Shaw's laboratory has explored differentiation of three different classes of cells, each of which is important to understanding regulated recruitment during immune responses: normal circulating leukocyte subsets, tumor infiltrating lymphocytes (TIL), and endothelial cells (9257). A simple and powerful strategy was devised for mAb analysis by flow cytometry on whole blood using humanized mAb to characterize in detail the surface phenotype of 7 circulating cell types for more than 100 different surface molecules. Among many specific findings, a striking overall picture emerges from this analysis that many of the molecules implicated in leukocyte endothelial interactions (such as LFA-1, CD44, L-selectin) are broadly expressed by most (or all) lineages of circulating cells. This indicates that the fine specificity of adhesion regulation during recruitment must be substantially accounted for by other elements, such as specificity in triggering (see section 2. Cell Biology of Immune Responses). These investigators have also performed an extensive analysis of the cell surface phenotype and endothelial binding capacity of TIL cultured from human melanoma. Compared with peripheral blood T cells, TIL expressed high levels of 5 integrins, 2 other adhesion molecules (including the skin homing molecule CLA), and several activation markers. TIL bound avidly in vitro to resting endothelium via integrin pathways and to cytokine-activated endothelium via both integrins and selectin-mediated pathways. These phenotypic and functional properties of TIL should have complex and important effects on their migration in vivo, which can now begin to be analyzed. Finally, understanding of lymphocyte binding to endothelium depends on information not only on lymphocytes, but also on endothelium. Therefore studies have been initiated on the regulation of cell surface molecules on endothelium and soluble factors produced by them. Results of these analyses demonstrate cytokine-regulated expression of BGP and potentially other CD66 family molecules on endothelial cells and suggest a possible role in leukocyte-endothelial interactions.

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 HIV+ individuals, SLE patients, and patients with untreated Hodgkins disease and prostrate cancer 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-τ. 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 identification of costimulatory molecules involved in T cell activation has been carried out in the laboratory of Dr. Hodes (9266). A mAb (GL1) was generated which identifies a molecule expressed on activated B cells, macrophages, dendritic cells, and activated T cells. GL1 precipitates from the surface of B cells highly glycosylated molecules of heterogeneous apparent molecular weight of 65-100 kDa, with a core size of 35 kDa after removal of N-linked sugars. GL1 identifies a ligand for the T cell activation molecule CTLA4. This ligand is distinct from the previously described B7 (now B7-1) and is designated as B7-2. B7-2 is encoded by a gene related to but distinct from that encoding B7-1. Anti-B7-2 mAb GL1 inhibited accessory cell-dependent responses of T cells in vitro and in vivo, indicating that B7-2 is functionally a costimulatory molecule for T cell-dependent immune responses.

In order to identify previously uncharacterized activation molecules expressed on lymphocytes, Dr. Hodes' laboratory has characterized mAbs 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.

The expression and function of the cell adhesion molecule CD44 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. 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. 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. PCR analysis was used to analyze the expression of different 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.

Dr. Shaw's laboratory has continued studies of the "adhesion cascade" by which T cells bind to endothelium during the process of recruitment (9257).

These investigators have found that two structurally different soluble mediators which appear to act as physiologic "triggers" of lymphocyte adhesion: hepatocyte growth factor (HGF) and macrophage inflammatory protein-1ß (MIP-1ß). Findings with HGF confirm and extend previous MIP-1ß studies by this laboratory with respect to the concept that proadhesive factors are presented on the endothelial cell surface by proteoglycan-binding. Of particular importance, these factors act on different T cell subsets and thereby may influence recruitment of specific T cell subsets. Functional studies of cultured tumor infiltrating lymphocytes indicate that their adhesion to endothelium was far greater than that of resting PBT and similar to the adhesion seen with phorbol ester-stimulated T cells. Since this would contribute to dysregulation of recruitment, strategies are being explored to restore TIL to a more physiologic state of regulated adhesion.

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 inosotol (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^a (mtv-7) superantigen-bearing APC. Using TCR transgenic mice it was further demonstrated that in mice expressing Mls^a as a self antigen, no Mls^a-specific response was induced in peripheral T cells; in contrast thymocytes did respond to self Mls^a 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 signaling 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. 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 $CD4^+CD8^+$ 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^+ thymic epithelium (9268). They found that CD4 molecules on the surface of $CD4^+CD8^+$ thymocytes are engaged in situ by Ia^+ 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 has found that the molecular basis for low TCR expression in developing $CD4^+CD8^+$ 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 $CD4^+CD8^+$ thymocytes (9268). 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. Consistent with these observations, it was also found that the amount of lck associated with CD4 in $CD4^+CD8^+$ thymocytes is markedly increased in major histocompatibility complex class II^- mice in which surface CD4 molecules are not engaged due to the absence of the intrathymic ligand for CD4. These studies demonstrate a novel function for an intracellular tyrosine kinase in the regulation of TCR distribution and expression in immature thymocytes. This concept was supported by additional studies performed using thymocytes from CD4 transgenic mice in which CD4 is markedly overexpressed without concurrent increases in p56 lck. It was found that, due to increased expression of CD4 molecules without associated lck molecules, the ability of CD4 crosslinking to activate lck molecules was impaired, resulting in increased TCR expresseion and decreased degradation of nascent CD3δ chains. These findings not only support a model of TCR regulation by CD4 mediated signals, but also suggest that any surface protein with the ability to bind lck might consequently affect thymocyte development.

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 $CD4^+CD8^+$ thymocytes: (i) enhanced lck tyrosine kinase activity; (ii) inhibited TCR expression; and (iii) inhibited differentiation of immature $CD4^+CD8^+$ 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.

Dr. Alfred Singer's laboratory has examined the role of oligosaccharide processing on survival and assemble of nascent TCR proteins within the ER and their associations with molecular chaperone proteins important in TCR assembly (9268). It was found that failure to remove glucose residues from core oligosaccharide chains of TCR proteins dramatically impairs TCR assembly due to : a) an increased degradation rate of TCRα protein; b) inhibiton of association of nascent TCRα and TCRβ chains with the molecular chaperone calnexin; and c) reduction in assembly of TCR$\alpha\beta$ complexes. These studies define a critical role for oligosaccharide processing in TCR assembly and potentially provide a molecular basis for accelerated degradation of TCRα proteins within the ER of normal immature thymocytes.

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.

S. Sharrow and colleagues have analyzed cell surface expression of multiple class I MHC molecules on murine epidermal Langerhans cells, the antigen presenting cells of the skin (9255). It was found that Langerhans cells differentially express products of distinct class I genes such that these cells express low amounts of some, but not all, class I MHC antigens. Differential surface expression of products of distinct class I MHC genes by Langerhans cells may have a profound effect on cutaneous immune responses.

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 the RBL mast cell tumor line with combinations of the lymphocyte granule components cytolysin (perforin), granzyme A and granzyme B Using tumor cells as targets, RBL transfectants expressing only the membrane pore-forming cytolysin gave a modest IgE-dependent cytotoxicity without target DNA breakdown, while transfectants expressing cytolysin and both granzymes together killed tumor targets with accompanying DNA breakdown. In these transfectants, granzyme expression correlates with cytolytic and nucleolytic potency, and optimal transfectants show cytotoxicity equal to cloned cytotoxic T cells. These results indicate that cells have an internal death pathway which is triggered by 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 cytsteine 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^+ 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^+ donors, as well as other activation responses limited by TcR-induced death. Because this cell death is blocked by radical scavengers as well as by the xanthine oxidase inhibitor allopurinol, TCR-induced reactive oxygen intermediates were sought and detected using dichlorofluorescein. These results suggest that such oxidant species are the immediate mediators of this T cell death.

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). The activity is mediated by $CD56^+$ T cells and NK cells. CD44 directed lysis arises in these cells 24-48 hours after addition of IL-2 to the medium. These same cells express high amounts of CD44 and function as targetable killer cells through CD3 and CD16 prior to activation, suggesting that IL-2 induces a coupling of CD44 to the killing machinery. These studies show that the adhesion molecule CD44 can serve as a cytotoxic triggering molecule on a subset of PBL and raise the questions of how CD44 on these cells differs from that on other cytotoxic cells, and what the biological function of this phenomenon 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. From previous studies of mice derived from ES cells multiply infected with a single retorviral vector, 4 proviruses have been identified that disrupt embryonic development when homozygous. The laboratory is currently focussing on one of these, 412-a, in an effort to identify and isolate the affected gene and to understand the

mutant phenotype (9297). The retroviral insertion site has been cloned and genetically mapped to chromosome 8. Specific regions that may represent part(s) or all of the mutated gene have been identified and isolated as subclones. Studies are underway to sequence these regions and use them to isolate cDNA clones. In addition to these molecular studies, the mutant phenotype is being analyzed at the level of the dissecting microscope and histologically.

For the past few years, Dr. Kuehn's laboratory has been studying the 413-d retroviral insertional mutation which causes a failure of gastrulation (9299). The gene identified by this mutation, nodal, is expressed in normal embryos at a time consistent with a role in mesoderm induction. In addition, it is expressed later in gastrulation around the node, a structure playing a critical role in the further induction and patterning of mesodermal structures in amniotes. Current studies have addressed whether nodal, a member of the TFG-beta superfamily of secreted growth factors, is indeed a mesoderm inducing and patterning factor. These studies have utilized Xenopus laevis embryos, into which defined amounts of nodal mRNA have been injected. These studies have shown that ectopic expression of nodal results in the formation of different mesodermal cell types depending on the amount of mRNA injected. The results suggest a role for nodal both in initial mesoderm induction as gastrulation starts, as well as in the determination of various mesodermal cell fates later in gastrulation.

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. This participation appears to involve the formation of pores in the membrane of the veto cell by precursor CTL-derived cytolysin. These findings suggest that there exists within the cytotoxic arm of the immune response an intrinsic mechanism for protection against attack directed at self antigens.

Dr. Gress' laboratory has further investigated the regulation of CTL in transplantation responses in vivo (9287) by studying the ability of Th2 cells to regulate graft-versus-host disease (GVHD). A murine parent-into-F1 model

was used to evaluate the effect of Th2 cells which had been generated by treating parental mice with IL-2 plus IL-4. Cell mixing experiments demonstrated that Th2 cells could in fact protect hosts from otherwise lethal cell inocula. This protection from lethality was associated with regulation of cellular- and cytokine-mediated events.

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 allogenic HLA-mismatched, T cell depleted allogeneic marrow and autologous marrow purged of malignant T cells in the treatment of aggressive hematolymphopoietic 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) by studying the in vitro cellular immune responses of T cells from patients who have received renal or cardiac allografts and who are on immunosuppressive drug protocols. It was found that rejection in these two situations is dependent on the recipient's antigen presenting cells and not on those of donor origin. Using this information, Dr. Shearer's group was able to predict non-rejections among renal and cardiac graft recipients with 90-95% accuracy. However, they overestimated their predictions for rejections by two-fold. Follow-up of renal graft recipients indicated that 90% of those predicted to be rejectors had lost their kidney grafts within three years of immunologic testing.

8. TUMOR IMMUNOLOGY

A mouse model for retargeting the immune system against syngeneic mammary tumors has been studied in the laboratory of Dr. Segal (9254). The model utilizes tumors, both primary and passaged, induced by the vertically transmitted mammary tumor virus. Cytotoxic T lymphocytes from normal mice have been retargeted with conventional and genetically engineered bispecific antibodies having specificity for murine CD3 and for the gp52 virus envelope protein, which is selectively expressed on the surface of the mammary tumor cells. 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. When injected iv into syngeneic mice, the 64PT mammary tumor line produces lung metastases which kill the mice within 2-4 weeks of injection. Ip injection of bispecific antibody immediately following tumor strongly blocks tumor growth in these mice.

In other studies, designed to improve targeting reagents, Dr. Segal's laboratory has genetically engineered sFv fusion proteins from anti-DNP and anti-tranferrin receptor (TfR) 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. Two sFvs, an anti-CD3 and an anti-huTfR construct, were linked together by a polypeptide spacer connecting the C terminus of anti-CD3 to the N terminus of anti-huTfR. This construct was secreted by COS cells, bound both CD3 and huTfR, and redirected T cells to specifically lyse target cells expressing huTfR. Thus, a single chain construct can be made that will redirect lysis. This procedure should greatly simplify the production of bispecific antibodies in the future.

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, 9254, 9287, 9288).

During the past year, the EIB flow cytometry laboratory (9255) has continued to develop flow cytometry resources. Modifications have been designed, produced and implemented to enhance throughput, functionality and flexibility of flow cytometry instrumentation and of associated computer hardware and software.

Dr. Shaw organized two components of the 5th International Workshop on Leukocyte Differentiation Antigens (9257). He performed two roles new to this

series of workshops: 1) information management and construction of a user-friendly information database; and 2) organization of the cross-lineage "blind" panel, a quantitative flow cytometric analysis of a comprehensive set of mAb against all known hematopoietic cell surface molecules. Both roles were key to the success of the overall workshop which was a massive undertaking (1500 mAbs, 400 labs, 800 participants, assignment of about 50 new molecules, conducted over the course of two years). Dr. Shaw designed a quantitative flow cytometric approach for the Blind Panel which was conducted using 465 selected mAb in 28 labs in 109 assays. The size, data quality and strategy of statistical analysis of the Blind Panel resulted in a rich source of "new" molecules which were given CD designations in the workshop. Furthermore, the information from those and other analyses was used to construct a "Leukocyte Differentiation Antigen Database" (LDAD). LDAD provides: 1) easy access to information on the 140 molecules and 1460 mAbs studied in the workshop; and 2) the ability to display/analyze quantitative expression of each molecule on the 80 different cell types tested. LDAD is being circulated widely both via INTERNET and mailing to more than 1000 interested individuals and is proving to be a valuable resource.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09251-23 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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
	R. Blumenthal	Microbiologist	EIB, NCI
	M. Cibotti	Visiting Fellow	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:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (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). With red cell targets, granzyme expression has no effect on lytic potency, which is higher than cloned CTL for all RBL-cy-x. On tumor targets, RBL-cy transfectants show only modest cytotoxicity, with no accompanying target nuclear damage. Both RBL-gza and RBL-gzb give significant target nuclear damage as well as enhanced cytotoxic potency compared to RBL-cy, and RBL-cy-gza-gzb show further enhancement of both properties. The latter are close (3x-4x fewer lytic units) to cloned CTL. Analysis of multiple RBL-cy-gz transfectant clones shows their cytotoxic potentcy is independent of cytolysin expression levels (in the range examined) but proportional to both granzyme A and granzyme B expression. In order to ascertain if granzymes act on a substrate inside the target cells, the protease inhibitor aprotinin was loaded into target cells using osmotic lysis of pinosomes. This was shown to block both lysis and nuclear damage by RBL-cy-gza and CTL, but not RBL-cy. Addition of aprotinin to the medium had no effect, implying granzymes act inside the target. To see if the nucleus is required for the granzyme-mediated death pathway, enucleated tumor target cells were compared to intact tumor cells and red cells. These cytoplast targets behaved like the intact tumor cells and not red cells, in that RBL-cy-gza-gzb killed them much better than RBL-cy. Thus we conclude that the nucleus is not involved in this death pathway, and that the physiologically relevant granzyme substrate responsible for cell death is cytoplasmic..

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. We have constructed triple, double, and single RBL transfectants expressing cytolysin (cy) and the granule serine proteases granzyme A (gza) and granzyme B (gzb). With red cell targets, granzyme expression has no effect on lytic potency, which is higher than cloned CTL for all RBL-cy-x. On tumor targets, RBL-cy transfectants show only modest cytotoxicity, with no accompanying target nuclear damage. Both RBL-gza and RBL-gzb give significant target nuclear damage as well as enhanced cytotoxic potency compared to RBL-cy, and RBL-cy-gza-gzb show further enhancement of both properties. The latter are close (3x-4x fewer lytic units) to cloned CTL. Analysis of multiple RBL-cy-gz transfectant clones shows their cytotoxic potentcy is independent of cytolysin expression levels (in the range examined) but proportional to both granzyme A and granzyme B expression. In order to ascertain if granzymes act on a substrate inside the target cells, the protease inhibitor aprotinin was loaded into target cells using osmotic lysis of pinosomes. This was shown to block both lysis and nuclear damage by RBL-cy-gza and CTL, but not RBL-cy. Addition of aprotinin to the medium had no effect, implying granzymes act inside the target. To see if the nucleus is required for the granzyme-mediated death pathway, enucleated tumor target cells were compared to intact tumor cells and red cells. These cytoplast targets behaved like the intact tumor cells and not red cells, in that RBL-cy-gza-gzb killed them much better than RBL-cy. Thus we conclude that the nucleus is not involved in this death pathway, and that the physiologically relevant granzyme substrate responsible for cell death is cytoplasmic.

Proposed course:

We are carrying out further experiments using RBL transfectants with cytoplast targets to confirm the generality of our findings. In collaboration with the laboratory of Dr. Pierre Golstein we are testing to see if cytoplasts are good targets for CTL variants which utilize the Fas pathway for target cytotoxicity. In collaboration with the laboratory of Dr. Ronald Gress we are further testing our double degranulation model for the long established veto phenomenon for CTL tolerance.

Publications:

Vanguri P, Lee E, Henkart P, Shin ML. Hydrolysis of myelin basic protein in myelin membranes by granzymes of large granular lymphocytes. J Immunol 1993;150:2431-2439.

Nakajima H, Henkart PA. Cytotoxic lymphocyte granzymes trigger a target cell internal disintegration pathway leading to cytolysis and DNA breakdown. J Immunol 1994;152:1057-1063.

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

PROJECT NUMBER
Z01 CB 09254-20 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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:	G. Sconoccia	Visiting Fellow	EIB, NCI
	T. Bakacs	Visiting Scientist	EIB, NCI
	C. Zacharchuk	Senior Investigator	DCT, NCI
	M.B. Moreno	Special Volunteer	DCT, NCI

COOPERATING UNITS (if any)
Cooperating Units: 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:	PROFESSIONAL:	OTHER:
2.0	1.0 1.0	

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☐ (a1) Minors
☐ (a2) Interviews
B

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

A murine breast cancer model has been developed for measuring the ability of targeted effector cells to eradicate primary and transplanted mammary tumors. A genetically engineered bispecific $F(ab')_2$ construct mediates redirected lysis of primary and cultured mouse mammary tumor cells, and blocks growth in Winn assays.

By using anti-CD44 containing bispecific antibodies, we have found that CD44 is a cytotoxic triggering molecule on a subset of human PBL. Both $CD56^+$ T cells and NK cells gain this activity between 24-48 hr stimulation with IL-2 or IL-12. The activity is generated in cells that express CD44 and mediate other types of lysis prior to activation, and may involve the coupling of the CD44 adhesion molecule to the lytic machinery.

Project Description

Major Findings

Breast 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 in collaboration with J. Tso, prepared a genetically engineered bispecific antibody (bsAb) consisting of 2C11 (anti-mouse CD3) Fab linked to P2AE12 (anti-gp52) Fab. Activated murine T cells targeted with this bsAb lyse primary mammary tumor cells and cultured cell lines and block the subcutaneous growth of tumor in syngeneic mice in a Winn-type assay.

This bsAb binds to T cells with an affinity of about $3x10^7$ M^{-1}, but binds to tumor cells with about 20 fold lower affinity. When radiolabelled and injected into mice, it homes well to lymphoid organs, but does not get to subcutaneous mammary tumor. Moreover, the intact anti-gp52 mAb, which binds with an affinity of 10^8 M^{-1} to tumor cells (due to divalent binding) also does not localize in the tumor to a significant extent, suggesting that in the subcutaneous environment, mammary tumor grafts are inaccessible to injected antibody. Nevertheless, treatment of mice with high doses of bsAb and IL-2 reproducibly delays the growth of mammary tumor grafts.

Targeting with CD44

Previous studies have shown that anti-CD44 mAbs mediate redirected lysis in mouse and human T cell clones. To establish whether CD44 is a cytotoxic trigger molecule in human PBL subsets we tested a bsAb consisting of anti-CD44 Fab crosslinked to a Fab against a target cell antigen for the ability to induce target cell lysis. We found that PBL were able to mediate targetable cytotoxic activity with the anti-CD44 bsAb provided that the effector cells were stimulated with either IL-2 or IL-12. CD44-directed cytolysis appeared between 24-48 hr after addition of IL-2; in contrast, CD3 redirected lysis and ADCC were both mediated by unstimulated cells. Cell fractionation experiments showed that CD44-directed cytolysis was mediated exclusively by $CD56^+$ low buoyant density cells, mainly by the $CD16^+$ (NK) subset of these cells, but also to a lesser extent by the $CD3^+$, $CD56^+$ subset. Unstimulated $CD56^+$ cells uniformly expressed easily detectable levels of CD44 that increased 40-50% upon stimulation with IL-2. No changes in isoform expression resulting from activation could be detected in the extracellular domain of CD44 using isoform-specific mAbs. Thus, lymphokine stimulation causes CD44 to become a cytotoxic trigger molecule in subsets of PBL that were cytotoxic and expressed CD44 prior to activation. The coupling of the CD44 adhesion molecule to the lytic machinery in these cells may be related to the generation of lymphokine activated killer (LAK) activity.

Proposed course of project

Mouse mammary tumor model

Because subcutaneous mammary tumor grafts are inaccessible to iv administration of bsAb, we will continue our studies using a lung metastasis model, and will try to block the onset of primary mammary tumors in C3H ex-breeder female mice. We have found that the 64PT tumor that we have used for in vitro studies, Winn assays, and homing studies localizes to the lung and forms metastases that are readily apparent 20 days after iv injection of tumor cells. We are currently giving bsAb at various times after tumor with varying doses of IL-2, to see if we can block the appearance of lung metastases. In order to determine whether the bispecific antibodies have an effect on the appearance of spontaneous tumors, we will treat C3H ex breeders with bsAbs under varying conditions, and compare tumor formation in treated and untreated animals.

CD44 targeting

Our next experiments will aim to 1) determine how CD44 becomes a cytotoxic triggering molecule in T or LAK cells, and 2) to determine whether CD44 is involved mediating lysis of target cells in the absence of bsAb. To determine how CD44 becomes a triggering molecule, we need to develop cloned NK or T cells that mediate CD44-directed lysis. These studies are currently underway. When we have a purified population of cells that mediates this activity we will compare CD44 on these cells with that on cells where CD44 does not trigger lysis. We will look for the expression of a new isoform, particularly in the cytoplasmic domain, for the association with other molecules, such as TcR Zeta and FcϵRI gamma chains, and we will look for modifications of CD44, for example in phosphorylation state. To determine whether CD44 is involved in triggering lysis in the absence of bsAbs, we will try to prepare a polyclonal antibody against CD44 and use it to block lysis. The monoclonal anti-CD44 antibody we have does not inhibit natural cytotoxicity mediated by LAK cells, but a previous study has shown that in the pig, NK activity could be inhibited by a polyclonal anti-CD44, but not by several monoclonal antibodies. We have already cloned cDNA encoding the extracellular domain of CD44, and will express it in bacteria. We will use this protein to immunize rabbits, and to affinity purify anti-CD44 from hyper-immune rabbit serum. This antibody will then be used in inhibition and targeting experiments.

Publications

Segal, DM, Jost, CR, and George, AJT.: Targeted cellular cytotoxicity. in Cytotoxic Cells: Recognition, Effector Function, Generation and Methods, M Sitkovsky and PA Henkart, eds. Burkhauser, Boston, 1993:96-110.

Daeron, M, Malbec, O, Latour, S, Bonnerot, C, Segal, DM, and Fridman, WH, Distinct intracytoplasmic sequences are required for endocytosis and phagocytosis via murine FcRII in mast cells. International Immunol 1993: 5:1393-1401

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

PROJECT NUMBER
Z01 CB-09255-20 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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)

L. Barden, R. Tate, J. Powell, CSL, DCRT; M.C. Udey, S.I. Katz, DB, DCBDC, NCI.

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.75	1.0	1.75

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☐ (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 and electronic cell sorting.

Dr. A. Singer and colleagues have continuted to utilize flow cytometry analysis in studies of the relationships between T cell receptor expression and intra-thymic 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 studies have been extended to include analyses of mechanisms of thymocyte differentiation and the requirements for intra-thymic reciprocal interactions between T cells and epithelial cells during thymocyte development. These efforts depend upon multi-color analysis and the ability to analyze low frequency events. 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 investigators also used TCR transgenic SCID mice to show that when only TCRβ chains were expressed, medullary thymic epithelial cells were immature and disorganized as in non-transgenic SCID mice. In contrast, expression of TCR$\alpha\beta$ induced maturation and organization of medullary thymic epithelial cells the presence of mature medullary thymocytes. 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. These studies confirm an obligatory role during thymus development for surface expression of fully assembled TCR complexes.

Dr. A. Singer and colleagues have also used flow cytometry in studies of thymocyte differentiation in mice with genetically altered TCR components. Analysis of zeta-deficient mice showed that zeta chain expression, while not absolutely required for T cell differentiation, is necessary for full T cell maturation and expansion. It was also shown that overexpression of zeta chain led to premature termination of activity of recombination activating genes, prevented productive rearrangement on TCRα and TCRβ, and blocked development of double positive thymocytes. These data suggest that zeta chain expression can regulate early thymic differentiation by controlling TCR gene recombination.

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. 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 been extended to characterize non-TCR mediated components important in early thymocyte differentiation. It was shown that progression through one cell cycle is obligatory during the transition of $CD4^+CD8^{lo}$ cells into $CD4^+CD8^+$ thymocytes and that this progression is specifically regulated by interactions with cortical epithelial cells. The critical ligand expressed by cortical epithelial cells was found to be TGFβ. These findings not only have identified novel mechanisms for regulation via both TCR and non-TCR signalling, but also have contributed to our understanding of early thymocyte development.

Additional studies by Dr. A. Singer and colleagues have used flow cytometry to characterize signals controlling the level of TCR expression in developing thymocytes. They have shown that CD4 interactions with thymic epithelium regulate TCR expression in thymocytes via p56 lck, a CD4-associated tyrosine kinase. They have also shown that the membrane bound protein tyrosine phosphatase CD45, known to regulate lck activity, may also play a role in thymocyte differentiation. Treatment of developing thymocytes (*in vivo* or *in vitro*) with anti-CD45 monoclonal antibody was found to: a) enhance lck tyrosine kinase activity; b) inhibit TCR expression; and c) inhibit the differentiation of CD4/CD8 double positive thymocytes into mature single positive T cells. These results suggest that CD45 may play an important regulatory role in thymocyte differentiation.

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 Vβ 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 Vβ-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.

These TCR repertoire studies by Dr. Hodes and colleagues have been extended to include analyses of TCR Vα/Vβ pairing. It has been found that Vα/Vβ pairing

is non random and that there exist strain specific differences in specific Vα/Vβ pairs. Furthermore, patterns of preferential Vα/Vβ pairing were expressed independently within Cd4 and CD8 T cell subsets within an individual strain. Therefore, the expressed Vα/Vβ repertoire appears to be a consequence of thymic selection rather than solely determined by structural constraints. These results suggest that detailed analysis of Vα/Vβ TCR pairs will provide a novel approach for investigation of T cell repertoire selection by self antigens.

Dr. Hodes and colleagues have also used flow cytometry in their identification and characterization of a new molecule which they have found to be a ligand for CD28. This new molecule, GL1 or B7-2, was shown to be expressed preferentially by activated T and B cells, including a subpopulation of freshly explanted thymocytes. It has also been shown that B7-2 can provide costimulatory signals to T lymphocytes and that a variety of stimuli can regulate B7-2 expression. These studies are important to our understanding of costimulatory signals important in B and T cell activation.

Dr. Ronald Gress and colleagues utilized flow cytometry in a series of studies of mechanisms of bone marrow tranpslantation 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 reconstituion, and to evaluate immunosuppressive therapies used to prevent graft rejection. Dr. Gress' laboratory has 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 populaitons. 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. Gene Shearer and colleagues utilized flow cytometry for characterization of the recovery of T cell populations after an acute graft-vs-host reaction in the mouse, a model which produces long term immune disfunction characterized by hyporesponsiveness. It was demonstrated that those T cells which reconstituted following an acute graft-vs-host reaction were derived from donor (graft) cells. These T cells were phenotypically and numerically normal, did not exhibit suppressive activity, and showed evidence of clonal deletion consistent with thymic processing. There results suggest that the immune deficiency following an acute graft-vs-host reaction may be due to shifts in the functional capacities of immune cells.

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. These investigations have demonstrated that functionally distinct T cell subsets express quantitatively different surface levels of multiple biologically functional molecules. Studies which focussed on the $CD4^+$ 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.

Dr. Shaw and colleagues have conducted systematic analyses of expression of 5 VLA integrin chains with simultaneous analyses of CD45RA and CD45RO isoforms which have demonstrated that regulation of VLA-α4 can occur independently of VLA-α3, VLA-α5, VLA-α6 and VLA-β1. It was also found that at least three subsets of memory cells can be discriminated on the basis of VLA-α4 expression (α4-negative, $\alpha4\beta$7-high, and $\alpha4\beta$1-high). One such subset has been identified which expresses high levels of $\alpha4\beta$7 and expresses phenotypic and functional characteristics of gut-homing cells. It has also been found that CD45RB defines two subsets of human memory $CD4^+$ T cells which interconvert depending upon activation status. 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 thes 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. More recently the VAX computers have served as the repository for the LDADS data base software developed by Dr. Shaw and available via INTERNET.

Dr. Mark Udey (Dermatology Branch, DCBDC, NCI) and colleagues have utilized flow cytometry to investigate the expression of cadhedrin molecules, known to be involved in keratinocyte-kerotinacyte interactions, on hematopoietic cells. They have demonstrated that murine epidermal Langerhans cells, the antigen presenting cells of the skin, synthesize E-cadherin and express this molecule on the cell surface. Because this was the first demonstration of expression of cadhedrins by hematopoietic cells, this study raised the novel possibility that cadhedrins may play a role in leukocyte migration. These investigators have also examined cadherin expression in developing murine thymocytes. It was found that thymocytes express E-cadherin in a developmentally regulated fashion and that E-cadherin was also expressed by both neonatal and adult thymic epithelial cells *in situ*. The results suggest that the homotypic adhesion molecule E-cadherin may play a role in developmentally regulated interactions between thymocytes and thymic stromal cells.

S. Sharrow and colleagues have analyzed cell surface expression of multiple class I MHC molecules on murine epidermal Langerhans cells using quantitative immunofluorescence. It was found that Langerhans cells differentially express products of distinct class I genes. Langerhans cells expressed low cell surface amounts of H-K and Qa-2, while expression of surface H-2D and H-2L by the same

cells was high. Murine epidermal Langerhans cells, therefore, express low cell surface amounts of some, but not all, class I MHC antigens. Differential surface expression of products of distinct class I MHC genes by Langerhans cells may have a profound effect on cutaneous immune responses.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09257-19 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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	J. Hallam	Biologist	EIB, NCI
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	M. Giunta	Visiting Fellow	EIB, NCI
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	E. Tolosa	Special Volunteer	EIB, NCI
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COOPERATING UNITS (If any)
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LAB/BRANCH
Experimental Immunology Branch

SECTION
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.5	7.5	

CHECK APPROPRIATE BOX(ES)

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

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

Our studies continue to emphasize characterization of cell surface molecules which facilitate T cell function, as well as elucidation of their regulation with T cell differentiation. We emphasize studies relevant to the regulation of leukocyte interactions with endothelium. Our studies with hepatocyte growth factor (HGF) and MIP-1β suggest that structurally diverse heparin-binding adhesion-inducing molecules are presented to leukocytes by proteoglycan on the endothelial lumen, and thereby selectively recruit different T cell subsets. Moreover, T cells appear to respond to HGF via a previously unidentified receptor distinct from c-met. We have characterized tumor infiltrating lymphocytes (TIL) in order to understand (and eventually optimize) recruitment of TIL administered as a tumor therapy. Their functional interaction with endothelium resembles that of activated T cells, and their phenotype is characterized by augmented expression of adhesion and activation markers. We systematically analyzed of expression of more than 100 molecules on 7 major subsets of circulating cells, and from that derived broad conclusions particularly regarding their contribution to selective leukocyte endothelial interactions. Furthermore, we have characterized regulation of cell surface molecules on endothelium and demonstrated cytokine-regulated expression of BGP and potentially other CD66 family molecules on endothelial cells, with presumptive relevance to leukocyte-endothelial interactions. Finally, I have organized a major international collaboration under the aegis of the Fifth International Workshop on Leukocyte Differentiation Antigens and from that generated a comprehensive database of information. I am disseminating it widely and it is proving to be a "extraordinary resource" to biologists.

Project Description

Major findings:

Our studies are directed at understanding the regulation of T cell function. We place particular importance on understanding the "antigen-independent" life of the T cell, which includes such essential functions as recruitment and migration into inflammatory sites. This has proved to depend on a far more elegant and complex molecular strategies that had previously been expected, which we believe reflects the importance of these elements in the orchestration of immune responses. We emphasize studies relevant to leukocyte interactions with endothelium. In general terms, our work supports and refines a "cascade" model which explains the rapid, efficient and selective interaction of lymphocytes with vascular endothelium, by a multistep process. This consensus model arising from work of ourselves and others is reviewed in more detail in a number of our recent publications (eg Lancet 343:831). We have been investigating intensively both the triggering step (whereby integrins on circulating leukocytes are rapidly activated) and the adhesion step (in which the activated integrins bind to endothelial ligands).

"Triggering" is a step in the leukocyte-endothelial adhesion cascade required to activate T cell integrins. Previous findings by ourselves and others implicate the chemokine family of cytokines as the best candidates for triggering molecules in lymphocyte recruitment. However, there must be diversity in the triggering step to account for the specificity of leukocyte subset-recruitment and it is unclear whether chemokines alone can provide enough diversity. We therefore explored whether other structural families of soluble mediators also can function as triggers. Our new evidence implicates hepatocyte growth factor (HGF). We find that HGF can induce both adhesion and migration of human T cell subsets and can be detected immunohistochemically on inflamed endothelium. HGF preferentially induces responses from T cells of memory phenotype, in contrast to the chemokine MIP-1β, which we have previously shown acts preferentially on naive cells. HGF, like the chemokines, binds to heparin and HGF retained in extracellular matrix is efficient in promoting migration. Furthermore, both MIP-1β and HGF induce actin polymerization within seconds, kinetics that approach those required to contribute to physiologic triggering. HGF is a member of a structural family distinct from the chemokines, whose only known receptor is the tyrosine kinase c-met. HGF induces tyrosine phosphorylation on T cells apparently via a distinct receptor since no c-met is detectable by surface staining, by PCR or by pTyr immunoprecipitation. Thus, promotion of T cell adhesion and migration are previously undescribed functions of HGF that we propose are relevant to selective T cell recruitment.

Integrins mediate leukocyte adhesion to vascular endothelium and thereby influence leukocyte recirculation. Regulated expression of specific integrin pairs on leukocyte subsets is an important means by which recruitment of those subsets is regulated. We have characterized expression by peripheral blood T cells of $\beta 1$ and $\beta 7$ integrins, particularly $\alpha 6\beta 1$ (VLA-6, CD49f), $\alpha 4B1$ (VLA-4, CD49d) and $\alpha 4\beta 7$ (LPAM-1). Integrin expression differs between $CD4^+$ cells and $CD8^+$ cells in that $CD4^+$ cells: 1) are more heterogeneous, particularly for $\alpha 4$; 2) express on the average less $\alpha 4$ and $\beta 7$; and 3) express on the average more $\alpha 6$ and $\beta 1$. 2D gel electrophoretic analysis was combined with flow cytometric analysis to determine which integrin chain pairs are expressed by the CD45RO- (naive) and CD45RO+ (memory) subsets of $CD4^+$ cells. CD45RO- (naive) cells express the three integrin pairs $\alpha 6\beta 1$, $\alpha 4\beta 1$ and $\alpha 4\beta 7$ at intermediate levels. Results for CD4+ CD45RO+ (memory) cells are more complex. Although 2D gel analysis demonstrates the same average integrin chain composition as CD45RO- $CD4^+$ cells, flow cytometric analysis demonstrates multiple subsets of CD45RO+ cells differing markedly from each other and from naive cells in levels of expression of $\alpha 6\beta 1$, $\alpha 4\beta 1$ and $\alpha 4\beta 7$. There are a minimum of three CD45RO+ subsets: 1) $\alpha 4\beta 1^{hi}\alpha 6\beta 1^{hi}\alpha 4\beta 7^{neg}$, which comprises the majority of the memory cells; 2) $\alpha 4\beta 7^{hi}\alpha 6\beta 1^{lo}$ presumptive gut-homing memory cells; and 3) $\alpha 6\beta 1^{hi}\alpha 4\beta 7^{neg}\alpha 4\beta 1^{neg}$, a previously unidentified subset expected to have unique migrational-functional properties. Of particular importance in these results are: a) the prominence/regulation of $\alpha 6\beta 1$ on $CD4^+$ cells; b) expression by $CD4^+$ naive cells of $\alpha 6\beta 1$, $\alpha 4\beta 1$ and $\alpha 4\beta 7$; and c) the selective decreases as well as increases in $\alpha 4\beta 7$ and $\alpha 4\beta 1$ during $CD4^+$ memory specialization.

We view the recruitment of specific leukocyte subsets to particular anatomic/inflammatory sites as a complex problem in information management. Evolution has devised a range of strategies to make sure that the information needed by leukocytes to decide whether they should emigrate is available at the endothelial surface. One strategy which we have elucidated is endothelial display of chemokines by retaining them on proteoglycans (Nature 361:79). This year, together with collaborator Art Anderson, we have formulated a concept of how these and other chemokines rapidly reach the endothelial surface via a specialized conduit system. The conduit consists of the fibroblastic reticular cell (FRC). For example, within a lymph node, the FRC conduit system is a meshwork of conduits which run from the site of entry of afferent lymph (the subcapsular sinus) to high endothelial venules (HEV), the site of lymphocyte entry. The characteristic microstructure of the FRC is consistent with a functional role in rapid movement of solute that occurs with minimal fluid volume and without dispersion due to fluid turbulence. Fluids appear to be pinocytosed from the afferent lymph, to move through the FRC conduit system and have a single predominant exit, the HEV. This specialized anatomic compartment is thus able to rapidly convey information from the lymph to HEV.

We have begun to apply our understanding of lymphocyte interaction with endothelium to a pertinent clinical problem: can we understand (and eventually optimize) the dynamics of recruitment of TIL (tumor infiltrating lymphocytes) when administered as a tumor therapy? The efficacy of cancer immunotherapy with cultured tumor infiltrating lymphocytes depends upon infused TIL migrating to tumor deposits where they can instigate an anti-tumor response. The specificity of TIL migration in vivo will be determined by their ability to bind selectively to tumor endothelium. In this context we carried out an extensive analysis of the cell surface phenotype and endothelial binding of TIL cultured from human melanoma. Compared with peripheral blood T cells TIL expressed high levels of 5 integrins, 2 other adhesion molecules, including the skin homing molecule CLA, and several activation markers. TIL bound avidly in vitro to resting endothelium via integrin pathways and to cytokine-activated endothelium via both integrins and selectin-mediated pathways. TIL adhesion to endothelium was far greater than that of resting PBT and similar to the adhesion seen with phorbol ester-stimulated T cells. We also compared the phenotype of TIL with allo-stimulated T cells that had been cultured under similar conditions. Despite the comparable culture conditions the two cell types differed in their expression of adhesion molecules and activation antigens. TIL expressed higher levels of the cutaneous lymphocyte antigen (CLA), the adhesion molecule CD31 and the activation markers CD30 and CD69 whereas several adhesion molecules and activation antigens were expressed at higher levels on allo-stimulated T cells. These characteristics of TIL might have conflicting effects on migration in vivo. For example, expression of CLA, the skin homing receptor, is likely to increase migration to melanoma (a skin cancer) whereas integrin activation is likely to result in non-specific binding of TIL to normal endothelium. We are now considering manipulating the culture conditions in which TIL are expanded in order to confer on them a phenotype which would be expected to be more conducive to selective tumor homing in vivo.

Given the complexity of the immune system, and the inter-relatedness of function of different molecules on the same cell, it is essential to systematically build an understanding of all molecules of the cell. One way in which such understanding has been generated and synthesized is the series of workshops designated the "International Workshops on Leukocyte Differentiation Antigens". These workshops have been successful in many ways, the most visible of which is the establishment of the standardized "CD" nomenclature. I assumed a major role in organizing the 5th International Workshop, which culminated in Boston in Nov 1993. The roles which I chose are ones never played before, which I thought were essential to the continuing evolution and success of the workshops: 1) information management; and 2) organization of the cross-lineage "blind" panel, a quantitative flow cytometric analysis of a comprehensive set of mAb against all known mAb. Both roles proved to be enormous ones, and to be essential to the success of surface molecules on hemotopoietic cells the massive undertaking (1500 mAbs, 400 labs, 800 participants, assignment of about 50 new molecules, conducted over the course of two years).

I designed the Cross-lineage Panel of the International Workshop to be as comprehensive and informative as possible. My objective was to co-ordinate a quantitative analysis of expression of "all" known cell surface molecules on as diverse a range of cell types as possible. Most of the analysis was done by flow cytometry because it is widely available, sensitive, flexible, quantitative and can be carefully standardized. Flow cytometry was conducted on a panel of 465 carefully selected mab in 28 labs in 109 assays. Thus, over 50,000 samples were stained in assays using more than 80 different cell types. The information derived from this extensive analysis was valuable to the workshop in assigning specificity to unknown mAb; even more important, it will be a resource to the scientific community in understanding the biology of these molecules. The quality of the design and data in the Blind Panel, together with its size and the strategy of statistical analysis, made it a rich source of "new" molecules which were given CD designations in this workshop. Specificially, it clustered 12 sets of "orphan" (unknown) mAb into groups that became new CDs.

My second objective in organizing the Cross-lineage Panel was to construct a body of information on tissue distribution of leukocyte which would be both comprehensive and easily accessible. Therefore, after compiling the information, I have made it available in two formats: a computerized database called the "Leukocyte Differentiation Antigen Database" (LDAD) and a printed version of critical excerpts of the database called the "Leukocyte Differentiation Antigen Encyclopedia". LDAD provides: 1) easy access to information on the 140 molecules and 1460 mAbs studied in the workshop; and 2) ability to display/analyze quantitative expression of each molecule on the 80 different cell types tested. I am circulating LDAD widely both via internet and via a mailing to more than 1000 interested individuals. Comments from recipients confirm that it is "an extraordinary resource" (Abul Abbas).

To broaden our understanding of the regulation of recruitment not only of T cells, but also of other circulating cell types, we undertook a comprehensive flow cytometric analysis of the cell surface phenotype of circulating cell types. We have devised a simple and powerful strategy for mAb analysis by flow cytometry on whole blood using humanized mAb to optimize both objectives. We have characterized in detail the surface phenotype of 7 circulating cell types with more than 450 mAbs of the Cross-lineage Panel of the 5th International Workshop on Leukocyte Differentiation Antigens. A striking "big picture" emerging from this analysis is how many of the molecules implicated in leukocyte endothelial interactions (such as LFA-1, CD44, L-selectin) are broadly expressed by most (or all) lineages of circulating cells. The implication of this finding is that the fine specificity of adhesion regulation must be substantially accounted for by other elements, such as specificity in triggering (see above). In addition, as a practical matter for subsequent workshops, we propose a technique we call "hierarchy analysis" as a novel approach to classify molecules and determine specificity of unknown mAb.

Understanding of lymphocyte binding to endothelium depends not only on an information on lymphocytes but also on endothelium. Therefore, we have begun to study the regulation of cell surface molecules on endothelium and soluble factors produced by them. Our most extensive study to date relates to CD66.

The carcinoembryonic antigen (CEA) gene family encompasses many members, some of which have been described as adhesion molecules and recently given the designation CD66. Among them are biliary glycoproteins (BGPs) which function on granulocytes to mediate binding to endothelial cells. We have found expression of CD66 on each of three types of cultured human endothelial cells: human umbilical vein endothelial cells (HUVEC), human dermal microvascular endothelial cells (HDMEC) and a transformed human microvascular endothelial cell line (HMEC-1). Basal CD66 expression varies from very low to intermediate, and is markedly induced by exposure to inflammatory cytokines. Interferon gamma (IFNγ) is the major inducer, but its effects are influenced by tumor necrosis factor-α (TNFα) and interleukin-1 beta (IL-1β). CD66 moAbs immunoprecipitate predominantly a 130-150 kD glycoprotein from cytokine-stimulated HUVEC and HMEC-1; further analysis on the HMEC-1 CD66 molecule shows that it is highly N-glycosylated, sialylated and contains O-linked oligosaccharides. The deglycosylated protein migrates in SDS-PAGE as prominent bands of apparent molecular mass of 60 and 70 kD and as a minor band of 50 kD. On HUVEC, the 70 kD band is phosphorylated. The pattern of binding of 45 CD66-related moAbs to endothelial cells correlates with those moAbs' specificity for the BGP molecule, now termed CD66a. PCR analysis confirms the presence of BGP message in stimulated endothelium. These findings demonstrate cytokine-regulated expression of BGP and potentially other CD66 family molecules on endothelial cells and suggest a possible role in leukocyte-endothelial interactions.

In addition to the foregoing studies, we are currently involved in several studies which are in their formative stages and from which we do not yet have definitive information: 1) characterizing the proteoglycans on lymphocytes and endothelium. Our prior studies suggest their important in regulation of localization of chemokines and others studies indicates their general relevance to adhesion. Therefore it is important to define the presence and function of proteoglycans on these cell types. 2) analyzing changes in actin in lymphocytes in response to chemical and other exogenous stimuli. Since actin is essential to adhesion/migration and since changes in actin rapidly "report" cellular response to stimuli, we expect it to be both informative as a reporter and a functional contributor to adhesive interactions. 3) Analyzing potential new receptors for CD31. Despite its recent popularity, ligands/receptors for CD31 are poorly defined. We are seeking to define such interactions using model systems with purified immobilized CD31.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09263-12 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Programmed cell death in lymphocytes

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

PI:	P.A. Henkart	Senior Investigator	EIB, NCI
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	R. Blumenthal	Microbiologist	EIB, NCI
	M. Williams	IRTA Fellow	EIB, NCI
	M. Clerici	Visiting Associate	EIB, NCI
	G. Shearer	Senior Investigator	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:	PROFESSIONAL:	OTHER:
3.5	3.0	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.)

We have continued investigating the protease-dependent programmed cell death (PCD) pathway triggered by the T cell receptor (TcR) in T hybridomas, activated peripheral T cells, and blood T cells from HIV+ donors. From the biochemical perspective we have shown that the calcium-dependent cytoplasmic protease calpain is activated in the T cell hybridoma 2B4 after TcR crosslinking, and that its activation is blocked by the protease inhibitors which block cell death. We have considered the hypothesis that calpain cleavage of xanthine dehydrogenase produces xanthine oxidase, which in turn generates reactive oxygen intermediates which kill the cell (a model previously described for neuronal excitotoxicity). In support of this, we found that the TcR induced death of both cell types was inhibited by radical scavengers, as well as by the xanthine oxidase inhibitor allopurinol. Further, in these cells TcR crosslinking was found to induce intracellular oxidation of dichlorofluorescin, which was blocked by N-acetyl-cysteine, allopurinol, and the cysteine protease inhibitor E-64. The thiol reagents and allopurinol did not act by preventing TcR signalling, since they did not inhibit TcR-induced IL-2 secretion in the hybridoma cells. Additional work has been devoted to defining the cell types in which this cell death pathway operates, and the influences of cytokines on this process. In both activated T cells and those from HIV+ donors, we have found that the "Type 1" cytokines IL-2, IL-12, and IFN-γ protect T cells from this cell death, and antibodies against the "Type 2" cytokines IL-4 and IL-10 also protect (implying that their endogenous production enhances cell death). These results suggest a model for HIV pathogenesis based on the Th1 to Th2 cytokine switch and with increasing antigen-triggered PCD as disease progresses.

Project Description

Major Findings:

We have continued investigating the protease-dependent programmed cell death (PCD) pathway triggered by the T cell receptor (TcR) in T hybridomas, activated peripheral T cells, and blood T cells from HIV^+ donors. From the biochemical perspective we have shown that the calcium-dependent cytoplasmic protease calpain is activated in the T cell hybridoma 2B4 after TcR crosslinking, and that its activation is blocked by the protease inhibitors which block cell death. We have considered the hypothesis that calpain cleavage of xanthine dehydrogenase produces xanthine oxidase, which in turn generates reactive oxygen intermediates which kill the cell (a model previously described for neuronal excitotoxicity). In support of this, we found that the TcR induced death of both cell types was inhibited by the radical scavengers N-acetyl-cysteine and mercaptopropionyl glycine, as well as by the xanthine oxidase inhibitor allopurinol. Further, in these cells TcR crosslinking was found to induce intracellular oxidation of dichlorofluorescin, which was blocked by N-acetyl-cysteine, allopurinol, and the cysteine protease inhibitor E-64. The thiol reagents and allopurinol did not act by preventing TcR signalling, since they did not inhibit TcR-induced IL-2 secretion in the hybridoma cells. In addition, these drugs did not inhibit the apoptotic death of the T hybridoma cells induced by corticosteroid, which in turn did not trigger oxidation of dichlorofluorescin. We propose that the TcR triggered death of activated mature T cells occurs via the calpain-xanthine oxidase-ROI pathway. We have also been seeking to define the cell types in which this cell death pathway operates, and the influences of cytokines on this process. Immature $CD4^+CD8^+$ thymocytes die after TcR crosslinking, but this death is not blocked by protease inhibitors. On the other hand, the TcR-induced death of $CD4^+8^-$ medullary thymocytes is sensitive to protease inhibitors. In activated T cells from normal human donors, as well as resting T cells from HIV^+ donors, TcR crosslinking causes death in both $CD4^+$ and $CD8^+$ subsets, all of which are blocked by protease inhibitors. In the latter cases, we have found that the "Type 1" cytokines IL-2, IL-12, and IFN-γ protect T cells from this cell death, and antibodies against the "Type 2" cytokines IL-4 and IL-10 also protect (implying that their endogenous production enhances cell death). Coupled with the increasing evidence for a cytokine profile shift in HIV^+ individuals progressing towards AIDS, these results suggest a model for AIDS pathogenesis in which the decline of $CD4^+$ T cells is due to increasing antigen-induced PCD as protective cytokines decline.

Proposed course:

Calpain activation is being measured under more conditions to establish the position of this step in the molecular pathway for TcR-induced PCD. Further experiments with different types of T cells, including those from HIV^+ donors, are being carried out to see if xanthine oxidase activity increases and cytoplasmic ROI are detectable under various conditions of TcR stimulation. Further experiments are being carried out on the cytokine dependence of antigen-induced PCD from normal T cells to test the hypothesis that TcR activation gives rise to both activation and death signals, with IL-2 and other endogenously produced cytokines normally blocking the death response.

Publications:

Sarin A, Adams DH, Henkart PA. Protease inhibitors selectively block T-cell receptor-triggered programmed cell death in a murine T cell hybridoma and activated peripheral T cells. J Exp Med 1993;178:1693-1700.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09264-07 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Studies of T Lymphocyte Function in Transplantation

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

PI:	G. M. Shearer	Section Chief	EIB, NCI
Other:	M. Clerici	Visiting Scientist	EIB, NCI
	R. Grady	Biotechnology Fellow	EIB, NCI

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LAB/BRANCH

Experimental Immunology Branch

SECTION

Cell Mediated Immunity Section

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2.5	1.5	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B, A, and D

SUMMARY OF WORK (Use standard unreduced 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 heterotopic heart transplant model involving immune suppression by cyclosporin A, we found a correlation between rejection and functionally 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 of the indirect pathway was indicative of a stable functioning graft and no 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 that 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, practical 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 functional. We found that doses of CsA that left the indirect pathway intact resulted in rejection, and was asssociated 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.

Studies of more than 120 blood samples from human cardiac transplants suggest that the indirect but not the direct pathway of recognition will be important in the rejection of cardiac allografts. Thus, 93% of patients whose PBMC exhibited a selective loss of the indirect T helper cell pathway did not show extensive lymphoid infiltrates. 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.

Publications:

Schulick RD, Weir MR, Miller MW, Cohen, Shearer GM. Longitudinal study of in vitro $CD4^+$ T helper cell function in kidney transplant recipients undergoing immunosuppression. Transplantation 1993;56:590-596.

Iwata H, Kitagawa S, Sato S, Kosugi A, Hirose H, Hamaoka T, Shearer GM, Fujiwara H. Suppression of allograft responses by combining donor alloantigen-specific i.v. presentization with suboptimal doses of FK506. Transplantation 1993;56:173-180.

Schulick RD, Muluk SC, Clerici M, Bermas BL, Via CS, Weir MR, Shearer GM. Value of in vitro $CD4^+$ T helper cell function test for predicting long term loss of human renal allografts. Transplantation 1994;57:480-482.

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

PROJECT NUMBER
Z01 CB 09265-12 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

PI:	R. J. Hodes	Senior Investigator	EIB, NCI
Others:	L. Palmer	Microbiologist	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: LTI, NCI
Villanova University
LIR, NIA, NIH

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

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 Vβ5, 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 Vβ2 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 Vβ14 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 Vβ-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 α and β chain components. Analysis of the expression of specific TCR Vα/Vβ pairs has indicated that Vα/Vβ pairing is non-random and that strain-specific differences exist in patterns of Vα/Vβ expression, providing a new approach to the study of repertoire selection. T cell responses to endogenous superantigen were also shown to be influenced by Vα as well as Vβ 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. Strain-specific decreases in expression occur in at least 12 of the 22 Vβ products in the process of eliminating T cells with potential reactivity for self determinants. A comparison of T cell receptor Vβ expression in congenic pairs of normal and athymic mice demonstrated that the normal Vβ deletions associated with tolerance to self products did not occur in athymic mice, indicating that the thymus has a critical role in mediating self tolerance by negative selection.

2) Analysis of ligands mediating Vβ-specific negative selection.

Endogenous mouse Vβ deleting ligands have been mapped to endogenous MMTV genes by several laboratories. Segregation analysis of deletions of Vβ5, 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 Vβ-specific deleting ligands in mice. To determine whether species other than the mouse express ligands for Vβ 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 Vβ 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 Vβ deletion.

3) Selective expression of specific Vα/Vβ pairing.

With the exception of the Vβ-specific recognition of superantigens, T cell recognition of antigen is generally determined by multiple TCR α and β chain segments. Selection of the T cell repertoire may therefore by detected by analysis, not of Vβ expression alone, but by expression of particular α chain/β chain pairs. An analysis of expression of specific Vα/Vβ pairs by T cells indicated that Vα's and Vβ's are not randomly associated on peripheral T cells. Moreover, patterns of Vα/Vβ 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 Vα expression, in addition to the dominant influence of Vβ 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 Vβ5 and Vβ11-bearing T cells. In vivo, at an early stage after viral infection, selective expansion and activation of $V\beta5^+CD4^+$ 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 Vβ products. A newly characterized tumorigenic MMTC in BALB/c mice (BALB/cV virus) was found to induce specific deletion of Vβ2-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 Vβ 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 Eα transgene. Deletion of Vβ14-bearing T cells required the expression of an I-E product. Moreover, susceptibility to viral infection as measured by levels or viral pp28 or MMTV LTR mRNA was also dependent upon transgenic I-E expression. These results suggest that I-E-dependent superantigenic stimulation of Vβ14 T cells plays a facilitating role in host infection with milk-borne MMTV. Preliminary results indicate that the incidence of mammary tumors in virus-exposed mice is significantly greater in animals expressing the Eα transgene.

Proposed Course of Research:

1) Analysis of ligands mediating Vβ-specific negative selection.

Mapping and transfection studies have identified a role of the MTV LTR gene in Vβ 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.

2) In vivo effects of exogenous retroviruses.

a) The relationship between MMTV-mediated Vβ specific-deletion and mammary tumorigenesis will be analyzed. Strains expressing class II MHC types or transgenic Vβ products that either do or do not support Vβ-specific deletion will be compared for susceptibility to MMTV infection and for mammary tumor incidence after exposure to milk-borne virus. TCR transgenics deficient in expression of MMTV superantigen-specific T cells will similarly be analyzed for susceptibility to viral infection.

b) In the MAIDS model of retroviral-induced immune deficiency, the nature of the observed T cell and B cell signaling abnormalities will be analyzed further, together with structural characterization of the TCR and sIg complexes and associated molecules.

Publications:

Abe, R and Hodes, RJ: Mouse endogenous superantigens: Mls and Mls-like determinants encoded by mouse retroviruses. In Coligan, JE, Kruisbeek, AM, Margulies, DH, Shevach, EM, and Strober, W (Eds.): Current Protocols in Immunology, John Wiley & Sons, New York, 1992: A.1.21-A.1.25,

Hodes, RJ, Abe, R, Gallahan, D, and Callahan, R: T cell receptor b-V repertoire expression in the absence of endogenous mouse mammary tumor provirus. Immunogenetics 37 1993:309-311

Hodes, RJ, Novick, MB, Palmer, LD, and Knepper, J: Association of a Vβ2-specific superantigen with a tumorigenic milk-borne mouse mammary tumor virus.J Immunol 150 1993:1422-1428

Pucillo, C, Cepeda, R, and Hodes, RJ: Expression of a MHC class II transgene determines both superantigenicity and susceptibility to mammary tumor virus infection.J Exp Med 178 1993:1441-1445

Selvey, LA, Morse, HC III, Granger, LG, and Hodes, RJ: Preferential expansion and activation of $V\beta5^+$ $CD4^+$ T cells in murine acquired immunodeficiency syndrome. J Immunol 151 1993:1712-1722

Shirai, M, Vacchio, M, Hodes, RJ, and Berzofsky, JA: Preferential Vβ usage by cytotoxic T cells crossreactive between two epitopes of HIV-1 gp160 and degenerate in class I MHC restriction. J Immunol 151 1993:2283-2295

Vacchio, MS, Kanagawa, O, Malissen, B, Tomonari, K, and Hodes, RJ: T cell receptor Vα-Vβ combinatorial selection in the expressed T cell repertoire. J Immunol 151 1993:1322-1327

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

PROJECT NUMBER
Z01 CB 09266-12 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

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Others:	K. Hathcock	Chemist	EIB, NCI
	H. Hirano	Visiting Fellow	EIB, NCI
	Q. Vos	Visiting Fellow	LIR, NIA
	Y. Yamashita	Visiting Fellow	LIR, NIA

COOPERATING UNITS (if any)
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

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☐ (a1) Minors
☐ (a2) Interviews

B

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-δ 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. A PCR-based approach has characterized tissue-specific expression of multiple CD44 isoforms resulting from alternative splicing of up to 10 exons.

mAb were 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 costimulus-dependent responses of $CD4^+$ T cells and identifies an alternative ligand for the T cell molecule CTLA4. The molecule identified by GL1 has been shown to be B7-2, the product of a gene related to but distinct from that encoding B7 (now B7-1). B7-2 product has been shown to play a predominant functional costimulatory role both in vivo and in vitro.

Project Description

Major Findings:

1) Identification of B7-2, a novel costimulatory molecule for T cell activation.

A mAb (GL1) was generated by immunization of a rat with activated B cells. GL1 identifies a molecule expressed on activated B cells, macrophages, dendritic cells, and activated T cells. GL1 precipitates from the surface of B cells highly glycosylated molecules with heterogeneous apparent molecular weight of 65-100 kDa, with a core size of 35 kDa after removal of N-linked sugars. GL1 identifies a ligand for the T cell activation molecule CTLA4. This ligand is distinct from the previously described B7 (now B7-1) and is designated as B7-2. B7-2 is encoded by a gene related to but distinct from that encoding B71. Anti-B7-2 mAb GL1 inhibited accessory cell-dependent responses of T cells in vitro and in vivo, indicating that B7-2 is a functionally costimulatory molecule for T cell-dependent immune responses.

Germinal center formation is a characteristic anatomic event during in vivo antibody responses in spleen, lymph node, and other lymphod tissues. Although germinal center formation is anatomically normal in aged animals, these animals fail to undergo somatic hypermutation of Ig genes, and hence fail to develop high affinity antibody responses. It has been observed that, in response to T-dependent antigens, germinal centers in young mice express high levels of B7-2 expression in the "dark zone" of germinal centers, where hypermutation is proposed to occur. In contrast, germinal center dark zones of aged mice fail to express B7-2. Expression of B7-2 costimulatory molecules during in vivo response thus correlates with somatic hypermutation and affinity maturation, suggesting that the defect in aged mice is related to failure to induce expression of costimulatory ligands.

2) 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. 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.

^{35}S-methionine metabolic labeling and ^{125}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. A mAb (H2Y2) was generated by immunization with fusion protein expressing CD44 variable exon products. This mAb reacts in a tissue-specific pattern with selected cells of epithelial origin as well as with discrete lymphoid cell subpopulations.

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 $CD4^+8^-$ 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 $CD4^+$ and $CD8^+$ 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.

4) Association of transgenic and endogenous Ig chains in Ig transgenic mice.

During characterization of Ig μ/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 μ chains associate in chimeric Ig molecules with endogenous μ or α chains produced by the same cell.

Analysis of transgenic mice expressing the same μ/k transgene on multiple genetic backgrounds revealed that expression of endogenous Ig was influenced by genetic background. These findings indicate that suppression of endogenous Ig expression (allelic exclusion) may be influenced by the products of a gene(s) that is polymorphic among inbred strains.

Z01 CB 09266-09 E

Proposed Course of Project:

1) Characterization of B7-2 costimulatory function.

The costimulatory molecule B7-2 appears to play a functionally predominant role in a number of in vivo as well as in vitro T cell-dependent responses. The effect of blocking this costimulatory pathway will be studied in vitro and in vivo in model systems including T-dependent antibody responses, allograft rejection, and T-dependent autoimmunity. The roles of alternative costimulatory ligands B7-1 and B7-2 will be compared.

Costimulatory molecules such as B7-2 may trigger signal transduction in T cells through interaction with receptors such as CTLA4 and CD28. B7-2 may also serve to directly transduce signals in B7-2-expressing cells as a result of B7-2 engagement. Both aspects of B7-2-dependent signaling will be analyzed.

The differential expression of B7-2 in germinal centers of young but not aged mice will be further analyzed to determine the nature of the defect in costimulus expression observed in aged animals.

2) CD44 expression and B cell activation.

The molecular basis for different functional properties of CD44, including hyaluronate (HA) binding by activated, $CD44^{hi}$ 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 on resting and activated lymphoid populations will be analyzed by PCR as well as with mAbs 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.

3) Identification of new B cell activation molecules.

Further functional characterization of the activation molecule recognized by GL7 will be carried out. Efforts have been initiated to identify gene encoding the GL7 target molecule by screening of an expression library constructed from activated B cell cDNA.

4) Analysis of allelic exclusion of Ig.

Genetic analysis will be pursued to identify the gene(s) influencing expression of endogenous Ig genes in μ/k transgenic mice. Initial efforts will distinguish whether strain-specific influences act on transgene expression or via modulation of the mechanism underlying allelic exclusion by the inhibition of endogenous Ig expression.

Publications:

Freeman, GJ, Boriello, F, Hodes, RJ, Reiser, H, Gribben, JG, Ng, JW, Kim, J, Goldberg, JM, Hathcock, KS, Laszlo, G, Lombard, LA, Wang, S, Gray GS, Nadler, LM, and Sharpe, AH: Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and IL-2 production. J Exp Med 178 1993:2185-2192

Freeman, GJ, Boriello, F, Hodes, RJ, Reiser, H, Hathcock, KS, Laszlo, G, McKnight, AJ, Kim, J, Du, L, Lombard, DB, Gray, GS, Nadler, LM, and Sharpe, AH B7 deficient mice reveal an alternative functional CTLA4 counter-receptor. Science 292 1993:907-909

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Hathcock, KS, Laszlo, G, Dickler, HB, Bradshaw, J, Linsley, P, and Hodes, RJ: Identification of a novel CTLA4 ligand that is costimulatory for T cell activation. Science 292 1993:905-907

Hirano, H, Screaton, GR, Bell, MV Jackson, DG, Bell, JI, and Hodes, RJ: CD44 isoform expression mediated by alternative splicing. Tissue-specific regulation in mice. International Immunology 6 1994:49-59

Laszlo, G, Hathcock, KS, Dickler, HB, and Hodes, RJ: Characterization of a novel cell surface molecule expressed on subpopulations of activated T and B cells. J Immunolo 150 1993:5252-5262

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09267-12 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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, laboratory, and institute affiliation)

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
	C. Chougnet	Fogarty Fellow	EIB, NCI
	Y. Yarchoan	Senior Investigator	DCT, NCI
	S. Broder	Director	DIR, NCI
	L. Pinto	Graduate Student	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., Seth Lederman, Columbia College of Medicine, NY.

LAB/BRANCH
Experimental Immunology Branch

SECTION
Cell Mediated Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B, A, D

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^+) 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 Type 1 over Type 2 function to a predominance of Type 2 over Type 1 function. Thus it maybe that Type 1 function is protective whereas Type 2 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%; and newborns of HIV-infected mothers, 35%. The majority of these individuals remains seronegative on follow-up although a few are PCR^+ for HIV DNA.

The studies summarized above raised the possibility that cellular immunity, mediated by Type 1 cells, is protective, but humoral immunity, mediated by Type 2 cells is not. If correct, these findings would indicate that AIDS vaccine development should be directed to augment cellular rather than humoral immunity.

We have also observed that interleukin-12 (IL-12) strongly enhances the defective in vitro Th responses of HIV^+ individuals, including HIV-specific responses. This finding raises the possibility of using IL-12 to enhance cellular immunity in HIV^+ patients.

Project Description

Major Findings:

The laboratory continues to obtain additional individuals within the cohorts of HIV-exposed, seronegative individuals whose T cells generate strong helper responses, and in some cases cytotoxic T lymphocyte activity, to HIV specific antigens. Thus from 35% 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. Approximately 25% of the HIV-exposed, T cell-reactive, seronegative health care workers exhibit HIV-specific cytotoxic T lymphocyte activity, which appears to be class I restricted. Approximately 17% of HIV-exposed, T cell-reactive, seronegative homosexual men are positive for proviral DNA. These two results suggest that at least a portion of these individuals have been infected with HIV. The results outlined above raised the possibility that cell-mediated but not antibody-mediated immunity is protective against HIV infection and/or progression to AIDS.

A longitudinal study of 355 of the asymptomatic HIV-seropositive (HIV^+) 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 10% of +/+/+, individuals progressed to AIDS in 3 years. 25% of -/+/+ and 50% 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^+ individuals is predictive for two relevant events in progression to AIDS -loss of $CD4^+$ cells, and death.

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-γ 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 support our hypothesis that the loss of T helper function in the progression toward AIDS is accompanied by a switch from a predominant Type 1 to a predominant Type 2 condition, and that a Type 1 response is protective against HIV infection and/or progression to AIDS, whereas a predominant Type 2 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 suggest that low dose immunization for the selective activation of potentially protective cellular immunity against HIV.

We have used the recently-discovered cytokine IL-12 to enhance in vitro cellular immune responses of HIV^+ individuals who exhibit immune defects. IL-12 restored Th function in response to recall antigens including to HIV antigens, without detectably affecting the cellular reponses of HIV^- control donors. These results raise the possibility that IL-12 could be used in therapy to restore Th function in HIV^+ patients.

In collaboration with Pierre Henkart and Apurva Sarin (EIB, NCI), we have tested the possibility that the increased programmed cell death (PCD) observed in HIV^+ individuals can be modulated with Type 1 and Type 2 cytokines. Thus Type 1 cytokines such as IL-12 and IFN-γ protect against PCD, whereas antibodies to these cytokines enhance PCD. In contrast, the Type 2 cytokines IL-4 and IL-10 do not protect against PCD, but antibodies against these two cytokines do provide protection. These results may be relevant mechanisms of immunopathogenesis involved in progression to AIDS.

Publications:

Clerici M, Yarchoan R, Blatt S, Hendrix CW, Broder S, Shearer GM. Effect of recombinant CD4-IgG on in vitro T helper cell function: data from a phase I-II study of patients with the acquired immunodeficiency syndrome (AIDS). J Infect Dis 1993;168:1012-1016.

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Clerici M, Lucey DR, Berzofsky JA, Pinto LA, Wynn TA, Blatt SP, Dolan MJ, Hendrix CW, Wolf SF, Shearer GM. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. Science 1993;262:1721-1724.

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Clerici M, Shearer GM, Hounsell EF, Jameson B, Habeshaw J, Dalgleish AG. Allocatived cytotoxic T cells recognize the carboxyterminal domain of human immunodeficiency virus-1 gp120 envelope glycoprotein. Eur J Immunol 1993;23:2022-2025.

Clerici M, DePalma L, Roilides E, Baker R, Shearer GM. Analysis of T helper and antigen-presenting cell functions in cord blood and peripheral blood leukocytes from healthy children of different ages. J Clin Invest 1993;91:2829-2836.

Clerici M, Wynn TA, Berzofsky JA, Blatt SP, Hendrix CW, Sher A, Coffman RL, Shearer GM. Role of interleukin 10 (IL-10) in T helper cell dysfunction in asymptomatic individuals infected with human immunodeficiency virus (HIV-1). J Clin Invest 1994;93:768-775.

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

PROJECT NUMBER
Z01 CB 09268-07 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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	IRTA Fellow	EIB, NCI
	Kelly Kearse	IRTA Fellow	EIB, NCI
	Ken Katz	IRTA Fellow	EIB, NCI

COOPERATING UNITS (if any)
LP, NCI; CBMB, NICHD; 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:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

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☐ (a1) Minors B
☐ (a2) Interviews

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

T cell receptor (TCR) expression and function in developing thymocytes was shown to be actively regulated by CD4-mediated signals generated by the interaction of CD4 with major histocompatibility (MHC) class II positive thymic epithelium. It was found that the molecular basis for low TCR expression in developing thymocytes is a high rate of degradation of newly synthesized TCR components, and that CD4 mediated signals regulate the TCR degradation rate in immature thymocytes via activation of tyrosine kinase p56 lck which was shown to be preferentially associated with CD4, rather than CD8. Indeed, the amount of lck associated with CD4 in immature thymocytes was shown to be inversely related to the extent of CD4 engagement by MHC class II molecules in the thymus. Overexpression of CD4 was found to interfere with the ability of CD4 crosslinking to activate associated p56 lck molecules and also to significantly inhibit CD4 regulation of TCR expression. 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. Biochemical analyses of TCR components demonstrated a critical role for oligosaccharide processing in the survival and assembly of nascent TCR proteins within the ER. It was found that persistence of glucose residues on core oligosaccharides: a) results in accelerated degradation of nascent TCRα proteins within the ER; b) prevents association of TCRα and TCRβ proteins with the molecular chaperone calnexin; and c) interferes with assembly of TCR$\alpha\beta$ complexes.

Project Description

Major Findings:

The role of CD4 signals in developing T cells has been investigated by biochemical characterization of CD4 and TCR components in several *in vivo* and *in vitro* models. It was found that *in vivo* CD4 cross-linking caused a 3-5 fold increase in surface expression of T cell receptor (TCR) on immature $CD4^+CD8^+$ thymocytes. It was also found that physical separation of immature $CD4^+CD8^+$ thymocytes from Ia^+ 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 (ER) 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, the phosphorylation status of TCR-zeta, a tyrosine kinase substrate, was examined in developing thymocytes. Consistent with the presence of a tonic CD4 signal in immature double positive thymocytes, it was 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. However, it was also found that TCR zeta chains are only associated with cell surface TCR and so cannot regulate assembly or transport of nascent TCR complexes.

To examine the mechanism of CD4-mediated regulation of TCR expression, the role of CD4-associated lck molecules in regulating TCR expression in immature $CD4^+CD8^+$ thymocytes and the activation of CD4-associated lck molecules by CD4 engagement were investigated. It was found that CD4 regulation of TCR expression in immature thymocytes is dependent upon the CD4-associated tyrosine kinase p56 lck, and that activation of lck in these cells results from engagement of surface CD4 molecules, but not CD8 surface molecules. It was shown that chronic engagement of CD4 activates associated lck molecules, which subsequently dissociate from CD4, generating "empty" CD4 molecules that are then internalized. The competence of CD4 to activate lck in $CD4^+CD8^+$ 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, providing a basis for the observation that CD8 engagement did not alter TCR expression of normal developing thymocytes. Consistent with these observations, it was also found that the amount of lck associated with CD4 in $CD4^+CD8^+$ thymocytes is markedly increased in major histocompatibility complex class II^- mice in which surface CD4 molecules are not engaged due to the abscence of the intrathymic ligand for CD4. These studies demonstrate a novel function for an intracellular tyrosine kinase in the regulation of TCR distribution and expression in immature thymocytes.

Similar studies were performed using thymocytes from CD4 transgenic mice expressing a CD4 transgene. T cells from these animals markedly overexpress CD4 without concomitant increases in p56lck and therefore allowed assessment of the consequences of increased numbers of "empty" CD4 molecules. It was found that overexpression of CD4 on $CD4^+CD8^+$ thymocytes impaired the ability of CD4 to activate associated lck molecules upon crosslinking, resulting in increased TCR expression and decreased degradation of nascent CD3δ chains. This paradoxical finding is most easily understood as the result of decreased lck aggregation during CD4 crosslinking (due to increased numbers of "empty" CD4 molecules)

$CD4^+CD8^+$ thymocytes, although it generates signals with consequences characteristic of positive selection, such as increased cell surface CD5. TCR signals failed to induce thymocyte apoptoses even when augmented by engagement with CD4 or LFA-1. However, signals generated by simultaneous engagement of TCR and the costimulatory molecule CD28 delivered a potent apoptotic signal, demonstrating that both TCR and costimulatory signals are necessary to induce thymocyte apoptosis. These results provide a molecular basis for differences among cell types in their ability to mediate negative selection of developing thymocytes.

Publications:

Love PE, Shores EW, Johnson MD, Tremblay ML, Lee EJ, Grinberg A, Huang SP, Singer A, Westphal H. T cell development in mice lacking the zeta-chain of the T cell antigen receptor complex. Science 261 1993:918-921

Punt JA, Osborne BA, Takahama Y, Sharrow SO, Singer A. Negative selection of $CD4^+CD8^+$ thymocytes by T cell receptor induced apoptosis requires a costimulatory signal that can be provided by CD28. J Exp Med 179 1994:709-713

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Van Ewijk W, Shores EW, Singer A. Cross-talk in the mouse thymus. Immunol Today 15 1994:214-217

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09273-07 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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:	Ricardo Cibotti	Visiting Fellow	EIB, NCI
	Shuichi Kubo	Visiting Fellow	EIB, NCI
	Jennifer Punt	Special Volunteer	EIB, NCI
	Joseph Roberts	BTP Fellow	EIB, NCI
	Harumi Suzuki	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)

DV, CBER, FDA; LMGD, NICHD; LC, DCE, NCI; Dept. of Biological Structure and Immunology, Univ. of Wash.; Paige Lab. of Dept. of Beterinary and Animal Sciences, Univ. of Mass.

LAB/BRANCH

Experimental Immunology Branch

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.0	5.0	1.0

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The relationship between T cell receptor (TCR) expression and T cell development and repertoire selection has been examined using a variety of in vitro and in vivo models. Studies on thymocytes from genetically defective scid mice have shown 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 nature of the TCR complex itself was studied using double TCR transgenic mice. It was demonstrated that each TCR/CD3 complex expressed on the surface of thymocytes contains precisely one TCR-alpha chain, one TCR-beta chain and two CD3-epsilon chains. The role of TCR components in T cell development was analyzed using zeta-deficient mice. These studies revealed that while zeta expression is not absolutely required for T cell differentiation, it performs a critical role in quantitatively promoting the generation of CD4/CD8 double positive thymocytes and is critical for the generation of mature single positive T cells. Studies of animals transgenic for different forms of zeta proteins demonstrated that overexpression of full length zeta chain terminated recombinant activating gene activity, prevented productive TCR rearrangement and blocked entry of thymocytes into the CD4/CD8 developmental pathway. 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 precursor thymocytes into double positive thymocytes and that this progression is specifically regulated by interaction with cortical epithelial cells and mediated by TGF-beta. Non-TCR mediated signals were also found to be critical in the process of negative selection of thymocytes. It was found that both TCR and costimulatory signals are necessary to induce thymocyte apoptosis and that CD28 can provide such a necessary costimulatory signal.

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. 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 T cell receptor (TCR) expression in T cell differentiation. It was found that $Thy1^+$ thymocytes from most scid mice contain only $CD4^-CD8^-$ (double negative) TCR^- cells which are similar to double negative cells from the thymi of normal mice. Introduction of TCR^+ cells into the thymi of scid mice promoted differentiation of scid thymocytes into CD4/CD8 expressing cells which themselves remained TCR^-. These studies have shown that T cells at different stages of development in TCRVβ8-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. Immunohistologic examination of the thymic stroma in scid mice demonstrated that while thymic medullary epithelium failed to organize and mature in the absence of TCR^+ cells, the introduction of TCR^+ cells into the scid thymus induced the normal maturation and organization of thymic medullary epithelium.

In order to directly assess the role of TCR^+ cells in the development of medullary thymic epithelial cells, mice were bred which coexpressed the scid genetic defect and transgenes encoding clonotypic TCR chains. It was found that medullary thymic epithelial cells from mice whose thymocytes only expressed TCRβ chains remained immature and disorganized. In contrast, medullary thymic epithelial cells from scid TCR$\alpha\beta$ transgenics were mature and organized. The ability of TCR^+ T cells to induce epithelial maturation was independent of antigen specificity, and induction of thymic medullary epithelial cells was associated with the presence of mature medullary thymocytes. These studies are important to our understanding of T cell differentiation and emphasize the critical nature of reciprocal interactions between thymocytes and thymic stroma in T cell and thymus development.

The nature of, and requirements for, TCR assembly and expression by the differentiating thymocytes themselves were studied using genetically altered mice. The TCR complex is composed of subunits which fall into three distinct groups of proteins: a) the disulphide-linked clonotypic subunits (α/β or γ/δ) which confer ligand binding specificity; b) the invariant CD3 chains, γ,δ,ϵ, which assembel as noncovalently linked heterodimers; and c) a disulphide-linked homo- or heterodimer composed of one or more members of the zeta family of proteins. The stoichiometry of the subunits comprising the T cell antigen receptor (TCR) complex was analyzed using mice transgenic for two different TCRα and two different TCRβ proteins. Individual thymocytes and splenic T cells from double TCR transgenic mice simultaneously expressed all four trangenic TCR proteins on their surfaces. It was found that while each TCRα chain paired with each TCRβ chain, each TCR complex contained only a single TCRα protein and a single TCRβ protein. In addition, it was found that

individual T cells expressed twice as many CD3ϵ proteins as TCRβ proteins. These findings demonstrate that each TCR/CD3 complex expressed on the surface of thymocytes contains precisely one TCRα, one TCRβ, and two CD3ϵ chains.

In other studies, the role of TCR components in T cell development was analyzed in zeta-deficient mice generated by gene targeting. It was found that zeta performs a previously unappreciated role in quantitatively promoting the generation and/or expansion of $CD4^+CD8^+$ thymocytes and is critical for the generation of CD4 or CD8 single positive mature T cells. However, small numbers of peripheral T cells expressing few T cell receptors were present in the periphery of these animals. These results show that while zeta chain expression profoundly influences thymocyte differentiation, it is not absolutely required for the generation of single positive T cells. The zeta family proteins (zeta, eta and gamma) each contain one or more copies of a conserved tyrosine-based activation motif (TAM) known to be required for signal transduction. To examime the developmental importance of multiple or individual TAM elements, transgenic mice expressing: a) full-length zeta chains (3 TAMS); b) eta chain, a naturally occuring variant (2 TAMS); or c) truncated zeta chain (1 TAM). It was found that overexpression of full length zeta chain caused premature termination of expression of recombination activating genes, prevented productive rearrangement of the TCRα and TCRβ genes and blocked entry of thymocytes into the CD4/CD8 developmental pathway. In contrast, overexpression of chains containing only 1 or 2 TAMS had no effect on normal thymocyte maturation. These results suggest that an early signaling pathway which regulates TCR gene recombination exists in precursor thymocytes and is differentially responsive to individual members of the zeta protein family.

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 $CD4^-CD8^{lo+}$ thymocytes which are the immediate precursors of $CD4^+CD8^+$ double positive thymocytes, a transition which occurs spontaneously in *in vitro* suspension culture. Studies of the requirements for this *in vitro* differentiation of murine $CD4^-CD8^{lo}$ precursor thymocytes into $CD4^+CD8^+$ cells were studied identified a post-transcriptional mechanism that is influenced by TCR signals and that regulates early thymocyte development. It was further found that intra-thymic negative selection of developing T cells can occur prior to the $CD4^+CD8^+$ 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 $CD4^-CD8^{lo}$ precursor thymocytes into $CD4^+CD8^+$ 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 β1 (TGF-β1) and TGF-β2. Thus, thymic epithelial cells expressing TGF-β proteins can actively regulate the rate at which $CD4^+CD8^+$ thymocytes are generated from $CD4^-CD8^{lo}$ precursor cells. 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.

The contribution of non-TCR mediated signals to thymocyte negative selection was also studied. Signaling requirements for the elimination of self-reactive T cells in the thymus were analyzed using *in vitro* induction of apoptosis. It was found that TCR engagement alone does not efficiently induce apoptosis of

resulting in inhibition of lck activation and consequently increased TCR expression. These findings further support the concept that TCR levels in immature thymocytes are regulated by CD4-mediated signals acting through lck, and suggest that any surface protein with the ability to bind lck might consequently affect thymocyte development.

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 $CD4^+CD8^+$ thymocytes: 1) enhances lck tyrosine kinase activity; 2) inhibits TCR expression; and 3) inhibits differentiation of immature $CD4^+CD8^+$ thymocytes into mature single positive T cells. These studies demonstrate that the ability of immature $CD4^+CD8^+$ 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 $CD4^+CD8^+$ thymocytes.

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.

These techniques were used to examine the role of oligosaccharide processing on survival and assembly of nascent TCR proteins within the ER and their associations with molecular chaperone proteins important in TCR assembly. Using an glucosidase inhibitor in mature T cells, it was found that the stability of newly synthesized TCRα, but not TCRβ or CD3, glycoproteins is markedly altered by the failure to remove glucose residues from core oligosaccharide chains. Persistence of glucose residues markedly inhibited associations of nascent TCRα and TCRβ glycoproteins with the molecular chaperone calnexin which is important for assembly of multichain complexes within the ER. It was further shown that the rapid degradation of nascent TCRα proteins induced by impaired glucose trimming severely limited assembly of TCR$\alpha\beta$ complexes. These studies define a critical role for oligosaccharide processing in TCR assembly and potentially provide a molecular basis for accelerated degradation of TCRα proteins within the ER of normal immature thymocytes.

Publications:

Wiest DL, Yuan L, Jefferson J, Benveniste P, Tsokos M, Klausner RD, Glimcher L, Samelson LE, Singer A. Regulation of T cell receptor expression in immature $CD4^+CD8^+$ thymocytes by $p56^{lck}$ tyrosine kinase: Basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. J Exp Med 178 1993:1701-1712

Nakayama T, Wiest DL, Abraham KM, Munitz TI, Perlmutter RM, Singer A. Decreased signaling competence as a result of receptor overexpression: Overexpression of CD4 reduces its ability to activate $p56^{lck}$ tyrosine kinase and to regulate TCR expression in immature $CD4^+CD8^+$ thymocytes. Proc Natl Acad Sci USA 90 1993:10534-10538

Kearse KP, Singer A. Isolation of immature and mature T-cell receptor complexes by lectin affinity chromatography. J Immunol Methods 167 1994:75-81

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

PROJECT NUMBER
Z01 CB 09279-09 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

P.I.:	Dinah Singer	Section Chief	EIB, NCI
Others:	Jocelyn Weissman	Chemist	EIB, NCI
	Kevin Howcroft	Staff Fellow	EIB, NCI
	David Nikodem	Chemist	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Experimental Immunology Branch

SECTION
Molecular 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 B
☐ (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 at varying levels. The 5' flanking DNA sequence of a swine class I gene region contains a series of negative and positive regulatory element. One of these elements, consisting of overlapping negative and positive regulatory elements, constitutes a regulatory domain responsible for establishing tissue-specific levels of MHC class I gene expression. The enhancer activity predominates in lymphoid tissues, but not in nonlymphoid tissues. This tissue-specific domain forms distinct enhancer and silencer associated complexes with cellular trans acting factors. Enhancer binding activity is present in all cell extracts. In contrast, the level of silencer binding is inversely proportional to the level of class I gene expression. These studies have led to our proposal that class I genes are 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 one subunit is also glycosylated. The silencer factor complex is approximately 95 kD. Extensive purification of these factors has been accomplished.

The only organ that does not express detectable levels of class I is the brain. A brain-derived neuroblastoma cell line normally does not transcribe class I genes. However, studies in our lab suggest that this is--at least in part--the result of active repression, mediated through the tissue specific regulatory element. Further analysis has revealed the existence of a brain-specific enhancer of class I gene 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, and absent in brain. 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, PD1, 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 PD1 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. Recent studies have focussed on class I expression in a neuroblastoma cell line. Like brain, this line does not normally express class I protein or RNA. However, class I transcription can be induced by interferon treatment of the cells. We have now demonstrated that the failure of these cells to express class I results from active repression of the promoter mediated by two silencer elements. One of these elements is the tissue-specific silencer described above, the other maps 3' to it. Both elements bind a common set of factors. In addition, a neuroblastoma-specific enhancer is revealed when the two silencer elements are removed.
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.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09281-08 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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, laboratory, and institute affiliation)

PI:	R. J. Hodes	Senior Investigator	EIB, NCI
Others:	K. S. Hathcock	Chemist	EIB, NCI
	L. Selvey	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)
LIR, NIA, NIH
Naval Medical Research Institute

LAB/BRANCH
Experimental Immunology Branch

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NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	

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☐ (a1) Minors

☐ (a2) Interviews

B

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, $[Ca^{2+}]_i$ was measured in response to surface receptor cross-linking. The $[Ca^{2+}]_i$ 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.

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 only minimally 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 multiple 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^a. Peripheral T cells from mice which express Mls^a were unresponsive to self Mls^a 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^a 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) 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:

Hathcock, KS, Hirano, H, and Hodes, RJ: CD45 expression by murine B cells and T cells: Alteration of CD45 isoforms in subpopulations of activated B cells. Immunol Res 12 1993:21-36

Kenny, JJ, Sieckmann, DG, Freter, C, Hodes, R, Hathcock, K, and Longo, DL.: Modulation of signal transduction in phosphocholine-specific B cells from μk transgenic mice.Curr Top Microbiol Immunol 1992:95-103

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

PROJECT NUMBER
Z01 CB 09282-08 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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. Mittleman	Biotechnology Fellow	EIB, NCI
	E. Mozes	Guest Researcher	EIB, NCI

COOPERATING UNITS (if any)
M. Petri, 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:	PROFESSIONAL:	OTHER:
3.2	2.2	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews
B, A and D

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^+) is also detected in patients with systemic lupus erythematosus (SLE). Thus, there are certain similarities between the immune dysregulation of SLE patients and HIV^+ 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^+ individuals can also been 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:

Via CS, Tsokos GC, Bermas B, Clerici M, Shearer GM. T cell-antigen-presenting cell interactions in human systemic lupus erythematosus: Evidence for heterogeneous expression of multiple defects. J Immunol 1993;151:3914-3922.

Hakim FT, Payne S, Shearer GM. Recovery of T cell populations after acute graft-vs-host reaction. J Immunol 1994;152:58-64.

Bermas BL, Petri M, Goldman D, Miller MW, Stocks NI, Via CS, Shearer GM. T helper cell depfunction in systemic lupus erythematosus (SLE): Relation to disease activity and interleukin 10 (IL-10) levels. J Clin Immunol 1994;14:169-177.

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

PROJECT NUMBER
Z01 CB 09285-08 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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,

P.I.	Dinah Singer	Section Chief	EIB, NCI
Others:	Lisa Palmer	IRTA	EIB, NCI
	Leonard Kohn	Senior Investigator	NIDDK
	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 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.7		

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☐ (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.)

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 thyrocytes. Thyrocytes normally express MHC class I, as does a rat thyrocyte 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. A TSH responsive element has been located within 68 bp upstream of the class I promoter, to a region which contains only canonical promoter elements. However, at least two additional elements, one of which is a CRE, are required for cAMP responses. Analysis of cell extracts from normal and TSH-treated thyrocytes reveals TSH-mediated differences in factors binding to these upstream elements.

Other agents are capable of modulating class I expression. Among them, insulin, hydrocortisone, methimazole, 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 elements responsive to thyroid hormone and hydrocortisone map to a segment between -135 bp and -209 bp, a region known to contain both the IFN-response element and enhancer A. Hydrocortisone has been demonstrated to alter the spectrum of factors associated with enhancer A, in particular NFκB. The site of action of methimazole, which has been shown to protect mice from experimental autoimmune disease, has been mapped to the DNA sequence element responsible for establishing tissue-specific levels of class I expression; methimazole induces changes in the DNA-binding factors associated with this DNA element.

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. 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.

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. It was demonstrated that a segment of DNA spanning 68 bp 5′ of the transcription initiation site was sufficient for TSH-mediated down-regulation. The only known DNA sequence elements contained within this region are the CAT and TATA boxes. Although the 68 bp segment is sufficient to confer TSH-sensitivity, additional regulatory elements appear to contribute to the overall response. Deletion of either a CRE-like element or a 30 bp regulatory element located between -135 bp and -177 bp eliminates the cAMP-mediated repression. TSH-sensitive changes in DNA binding complexes occur the involve both of the upstream elements, but changes in complexes within the 68 bp region have not been reproducibly detected.

Class I expression is also regulated by a variety of other agents, including hydrocortisone and methimazole. Both 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. We have now demonstrated that hydrocortisone acts by altering the

function of enhancer A; this is correlated with changes in complex formation in a gel shift assay in response to hydrocortisone. Methimazole has a distinct site of action, namely at the DNA sequence element responsible for establishing tissue specific levels of expression. This element consists of an overlapping enhancer and silencer, that together modulate class I expression. Methimazole affects the pattern of complexes associated with the silencer moiety.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09287-07 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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
	Daniel 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:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human ☐ (b) Human tissues ☒ (c) Neither B,D

☐ (a1) Minors

☐ (a2) Interviews

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 cell populations able to suppress host CTL responses 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. Specifically, triggering of veto cells was found to be mediated by target cell activation. In other studies, the in vivo regulatory role of donor Th2 cytokine type cells was evaluated. Such cells were found to modulate transplantation responses in vivo with effects on cytokine profiles and cell populations, and corresponding protection from graft versus host disease, in an animal model.

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}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 has been previously 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, might 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 showed that the precursor CTL, in interacting with veto cells, play an active role in their own clonal elimination. This role appears to be mediated by the release of cytolysin from recently stimulated precursor CTL and pore formation in the membrane of the veto cells with subsequent degranulation and lysis of the target precursor CTL.

With the demonstration that CTL are actively involved in transplantation responses in vivo, the information that CTL are dependent, in part, on Th1 responses, and the evidence that Th2 cells can regulate Th1 cells, we evaluated whether Th2 cytokine type cells might regulate transplantation responses in vivo. A murine parent-into-F1 graft-versus-host-reaction (GVHR) model that involves LPS-induced lethality was used for these studies. Parental cells of Th2 cytokine phenotype were generated by treating B6 mice with a combination of IL-2 and IL-4. Cell mixing experiments demonstrated that Th-2 type cells protected F1 hosts from LPS-induced lethality. Compared to control animals not receiving protective Th2-type cells, these animals had lower levels of CVD8+ donor cell engraftment, in vivo suppression of INF-γ mRNA, in vivo augmentation of IL-4 mRNA, and reduction of serum levels of TNF. Donor cells of Th-2 cytokine phenotype therefore prevent LPS-induced lethality during GVHR, and this protection is associated with regulation of cellular- and cytokine- mediated events.

Publications:

Cohen PA, Kim H, Fowler DH, Gress RE, Jakobsen MK, Alexander RB, Mule JJ, Carter C, Rosenberg SA. Use of interleukin -7, interleukin-2, and γ-interferon to propagate CD4+ T cells in culture with maintained antigen-specificity. J Immunotherapy 1993:242-252.

Fowler DH, Kurasawa K, Husebekk A, Cohen PA, Gress RE. Cells of Th2 cytokine phenotype prevent LPS induced lethality in murine GvHR: Regulation of cytokines and CD8+ lymphoid engraftment. J Immunol 1994;152:1004-1013.

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

PROJECT NUMBER
Z01 CB 09288-07 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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 Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) Minors
☐ (a2) Interviews

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 raised the possibility that residual T cells in infused T cell-depleted marrow play a central role in the generation of subsequent T cell populations. This possibility was confirmed in murine studies in which three T cell progenitor pools were identified which contribute to final T cell repopulation following marrow transplantation. It was found that cells arising from the peripheral, mature lymphocyte precursor pool were of memory phenotype, and that only T cells generated by a thymic pathway contained large numbers of naive T cells. The functional capacities of 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 presention 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 x 105/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 purging.

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 reconstition. 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 exogeneous 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:

Gress R, Katz SI, Lucas PJ. Human CD8+ xenoreactive T cells mediate tissue injury in vivo. Transplantation 1993;56:484-486.

Quinones RR, Gutierrez RH, Dinndorf PA, Gress RE, Ney A, Karandish S, Carter CS, Luban NLC, Reaman GH. HLA disparate bone marrow transplantation following T cell depletion by extended cycle elutriation. Blood 1993;82:307-317.

Mackall CL, Granger L, Sheard MA, Cepeda R, Gress RE. T cell regeneration after bone marrow transplantation: Differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. Blood 1993;82:2585-2594.

Yeoman H, Gress RE, Bare CV, Leary AG, Boyse EA, Bard J, Shultz LD, Harris DT, DeLuca D. Human bone marrow and umbilical cord blood cells generate $CD4^+$ and $CD8^+$ single-positive T cells in murine fetal thymus organ culture. Proc Natl Acad Sci USA 1993;90:10778-10782.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09289-05 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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
	C. Jost	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)
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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:	PROFESSIONAL:	OTHER:
3.5	3.5	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews
B and 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. Active sFv proteins have been produced in bacteria and in COS cells. In bacteria, a new procedure for refolding has been developed that dramatically increases the yield of active sFv. In COS cells, sFvs are secreted directly into the medium, and the rate of secretion is enhanced by inclusion of a glycosylation site. By genetically linking an anti-CD3 sFv with an anti-TfR sFv, a single chain bispecific antibody has been expressed by COS cells that mediates redirected lysis of TfR^+ target cells.

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 blocks antigen presentation to 2B4 cells and to other cells as well, at μM concentrations.

Project Description

Major findings

sFv 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.

We have generated several sFv clones including 2C11 (anti-mouse CD3), OKT9 (anti-human transferrin receptor) and U7.6 (anti-DNP). These sFvs were first expressed in bacteria giving large amounts of pure sFv which was insoluble and inactive. By solubilizing this protein in sodium lauryl sarcosine, forming disulfide bonds by air oxidation, and dialyzing against 0.4 M arginine, yields of active protein that approach 50% were obtained, a great improvement over the commonly used methods of refolding. All of these sFvs contained a peptide tag at their C terminal ends, and in the presence of an anti-CD3 x anti-tag bispecific antibody (bsAb), the OKT9 and U7.6 sFvs mediated redirected cytolysis.

sFvs were also produced in COS cells, in order to use the mammalian protein folding machinery to produce active protein. We transfected COS cells with plasmids encoding three different sFvs, 2C11, OKT9, and U7.6. Pulse-chase experiments showed that all four sFvs were synthesized by the transfected cells but that their secretion rates differed considerably. The rate limiting step of secretion was their exit from the endoplasmic reticulum in all cases. We found that N-linked glycosylated antibody sFvs were secreted faster than their non-glycosylated counterparts, and demonstrated that introduction of a glycosylation site increased secretion rates. All antibody sFvs specifically bound their antigens when secreted into the medium and where tested, at least 90% of the secreted sFv was functional. In experiments using glycosylated and non-glycosylated forms of U7.6 (anti-DNP) sFv, we found that the glycosylated form was folded into active antibody more rapidly than the non-glycosylated form, in the ER. These results show that sFv proteins, which differ in structure from intact native IgG, can nevertheless be processed normally by mammalian cells.

More recently, we have made a construct in which the C-terminal portion of 2C11 sFv is linked to the N terminus of OKT9 sFv using a 22 residue spacer. The resultant protein is secreted rapidly by COS cells, and has both anti-CD3 and anti-TfR binding activity. This single chain bsAb is able to redirect activated mouse T cells to lyse human TfR^+ cells.

T Cell Receptor sFv

A single chain Fv from the 2B4 T cell receptor (TCR) was constructed by linking the C terminus of V_α with the N terminus of V_β through a $(gly_4ser)_3$

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 to other hybridomas. Blockage of antigen presentation occurs in the μM range, presumably by binding to the MHC-II/peptide complex on the APC.

Current and future plans.

sFv-antibodies

We will continue our studies with single chain bsAbs to investigate their stabilities and biological properties. We will try to produce these antibodies in bacteria, to obtain high amounts of protein. This could provide a method for producing large amounts of bispecific antibody for clinical use.

We will also continue using sFvs to study their refolding in the ER. We plan to use crosslinking studies to see if the sFvs are associated with other proteins, for example chaperonins, in the ER, and if N-linked glycosylation has an effect on these interactions.

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 suffers 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.

Publications

1. Kurucz, I, Jost, CR, George, AJT, Andrew, SM, and Segal, DM.: A bacterially-expressed single chain Fv construct from the 2B4 T cell receptor. Proc. Nat. Acad. Sci. USA. 1993:90:3830-3834

2. George, AJT, Titus, JA, Jost, CR, Kurucz, I, Perez, P, Andrew, SM, Nicholls, PJ, Huston, JS, and Segal, DM. Redirection of T cell-mediated cytotoxicity by a recombinant single-chain Fv molecule. J. Immunol 1994: 152:1802-1811

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09297-03 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Characterization of a developmental mutation caused by retroviral insertion

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

PI:	M. Kuehn	Expert	EIB, NCI
Others:	E. Purpus	Fellow	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

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☐ (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.)

To identify and isolate developmental genes, we have been carrying out insertional mutagenesis studies in transgenic mice derived from retrovirally infected embryonic stem (ES) cells. We have previously reported the analysis of the 412 strain segregating 23 proviruses, 3 of which cause recessive prenatal lethality. The molecular and phenotypic analysis of the 412-k insertional mutation is currently underway.

Project description

Major findings:

Genetic analysis of the 412 strain was carried out to determine whether any of the 23 proviruses introduced into the germ line by ES cell mediated transgenesis cause insertional mutations in developmentally important genes. This was done by crossing animals heterozygous for a particular provirus, and examining the offspring for homozygotes. Of the 23 proviral insertions tested, 3 were never found in the homozygous state in live-born progeny. It was therefore assumed that these 3 proviruses cause recessive prenatal lethal insertional mutations, which potentially identify developmentally interesting genes. We are now analyzing one of these, 412-k, at the molecular and phenotypic levels.

At the molecular level, we have isolated the 412-k proviral integration locus as a set of overlapping cosmid clones. The provirus carries the bacterial neomycin/kanamycin resistance gene. Therefore it was possible to select these cosmids directly by plating the cosmid library on kanamycin. We then analayzed the cloned DNA for regions that may represent part or all of the mutated gene. This was accomplished by creating a sublibrary of random fragments from the cosmid inserts and screening it for clones that contain only unique sequence DNA. Four clones were derived each of which detects a different single restriction fragment when used to probe Southern blots of genomic mouse DNA. In addition, 2 of the subclones also hybridize to DNA of other organisms, suggesting that they might represent exons of an evolutionarily conserved gene.

So far, one of the subclones has been fully sequenced and the remaining 3 will be sequenced soon. The sequencing may reveal features that also suggest whether a clone represents part of a gene. These clones also will be used to screen specific cDNA libraries as determined by phenotype analysis described below.

At the phenotypic level, we have initiated an in depth visual and histological analysis of mutant embryos. Females and males heterozygous for 412-k are being mated and embryos are being collected on the 6th through the 10th day post coitum (dpc). These litters are being visually examined to determine abnormalities at each of these embryonic stages. By the end of the reporting period we will have determined the earliest sign of abnormalities and will have undertaken a screening of a cDNA library made from embryos of that stage using the probes described above.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09299-02 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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

Ectopic expression of the nodal gene in Xenopus laevis

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

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Other:	L. Lowe	Biologist	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

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☐ (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.)

We previously reported the isolation of the mouse nodal gene, a novel member of the TGF-beta superfamily. The gene was identified by a retroviral insertional mutation that causes embryonic arrest prior to mesoderm formation. To further characterize the gene we carried out studies to determine the effects of ectopic expression in another biological system, Xenopus laevis. These studies strongly suggest a role for nodal in mesoderm induction and patterning during axial development.

Project description

Major findings:

As reported previously, phenotypic analysis of embryos homozygous for a retroviral insertional mutation in the mouse nodal gene revealed abnormalities in gastrulation. Mutant embryos were characterized by a lack of embryonic mesoderm, showed no evidence for a primitive streak and had increased numbers of ectodermal cells. Our interpretation was that the mutation prevents the normal differentiation of ectoderm into mesoderm at this critical stage of embryonic development. The nodal gene encodes a novel member of the TGF-beta superfamily. A subset of members of this growing gene family have been shown to have mesoderm inducing activity in in vitro and in vivo assays utilizing Xenopus laevis embryos. To further characterize the nodal gene, we utilized these assays to determine whether nodal can function directly as a mesoderm inducing factor.

We first constructed a full length cDNA for the nodal gene using PCR. This was introduced into the standard SP6 based vector for in vitro transcription of the gene. Different amounts of the in vitro transcribed RNA was injected into Xenopus laevis fertilized eggs and the consequences of ectopic expression were assayed by examing intact gastrula stage embryos and animal caps isolated at the blastula stage. Animal caps will differentiate into skin if isolated away from the rest of the embryo at the early blastula stage. However, ectopic expression of certain growth factor genes will cause some of the animal cap cells to differentiate into mesoderm. This can be assayed by examination of the intact animal cap morphology, histological examination and analysis of expression of endogenous genes that are molecular markers of the different mesodermal cell types.

Examination of intact embryos injected with the highest levels of nodal RNA revealed normal development until early gastrula stages, at which point they exhibited altered morphogenetic movements and failed to develop a recognizable anterior-posterior axis. The phenotype resembled that of embryos treated with lithium chloride, suggesting that ectopic nodal expression hyperdorsalizes the embryo. Histological examination of the embryos revealed the presence of extensive amounts of notochord and somites. Molecular analysis demonstrated an increased level of expression of goosecoid and actin, further corroborating the overall dorsal-anterior development of embryos ectopically expressing nodal. Animal cap explants from embryos injected with the highest levels of nodal RNA exhibited a similar phenotype. The explants underwent extensive elongation, while control caps stayed round. Histological examination confirmed that ectopic expression of nodal diverts the animal cap cells from their normal epidermal fate; extensive amounts of notochord and muscle were seen. The expression of several genes which serve as mesodermal markers were assayed: Xbra a marker for all early mesodermal cell types, muscle actin a marker of the paraxial mesoderm and goosecoid which marks the most dorsal and anterior cell types. Only the highest levels of injected nodal RNA resulted in expression of goosecoid, while the other markers were present at lower levels.

These studies strongly suggest a role for nodal in mesoderm induction at the start of gastrulation. In addition, the dose dependant induction of different mesodermal cell types suggests a role for nodal in patterning of cell fate as gastrulation proceeds.

Publications:

none

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09400-02 EIB

PERIOD COVERED

October 1, 1993 to September 30, 1994

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	Senior Investigator	EIB, NCI
Others:	Leonard Kohn	Senior Investigator	NIDDK
	Edna Mozes	Guest Researchers	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

Molecular Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.5		

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☐ (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.)

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 thyroid stimulating hormone 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, we have previously shown that in an experimental model of autoimmunity, animals that fail to express class I molecules are resistant to disease. We have now further demonstrated that pharmacological agents that reduce class I in normal mice are protective against experimental systemic lupus erythematosus.

Project Description

Major Findings:

Experimental systemic lupus erythematosus (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, clinical manifestations of SLE develop: leukopenia, proteinuria, and immune complex deposits in the kidney. In contrast, following immunization with 16/6Id, class I-deficient mice generated anti-16/6Id antibodies, but did not generate anti-DNA or anti-nuclear antigen antibodies. Furthermore, they did not develop any of the clinical manifestations associated with disease. These findings indicated a major role for MHC class I molecules in the induction of autoimmune disease and suggested that agents which reduce MHC class I expression might mitigate experimental SLE in normal mice.

Studies in our lab had demonstrated that methimazole, a drug frequently used in the treatment of the autoimmune thyroid disease, Graves' disease, reduced transcription of MHC class I genes in thyrocytes. Therefore, we tested the effect of in vivo MMI treatment on the induction of experimental SLE in mice. MMI treatment of 16/6Id-immunize mice was found to reduce cell surface expression of MHC antigens on peripheral blood lymphocytes (PBL) and to alter the proportion of lymphocyte subpopulations. Moreover, MMI treatment of 16/6Id-immunized mice, initiated after the appearance of anti-16/6Id and anti-DNA antibodies, prevents the appearance of disease sequelae, including immune complex deposits in the kidney and blepharitis. These findings provide a possible new approach to the treatment of SLE.

Publications:

Mozes E, Kohn LD, Hakim F, Singer DS. Resistance of MHC Class I-Deficient Mice to Experimental Systemic Lupus Erythematosus. Science 1993;261:91-93.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09401-02 EIB

PERIOD COVERED: October 1, 1993 to September 31, 1994

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	Senior Investigator	EIB, NCI
Others:	T.K. Howcroft	Staff Fellow	EIB, NCI
	Julie Brown	Staff Fellow	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Experimental Immunology Branch

SECTION
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.3		

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☐ (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 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 is mediated by the HIV-1 Tat protein derived from a spliced viral transcript (two-exon Tat), identifying a novel activity for two-exon Tat, distinct from the transactivation of the HIV LTR common to both one-exon and two-exon Tat. Tat represses class I transcription by acting on the class I promoter. Mapping of functional domains of the Tat protein demonstrates that repression and activation are mediated by distinct domains.

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. It was found that the two-exon form of Tat, p16, which is derived from a fully spliced viral genome, is capable of significantly decreasing class I transcription. This effect on initiation occurs in the absence of the viral TAR sequence and is promoter specific. These findings establish a novel role for Tat; and for two-exon Tat in particular since one-exon Tat does not affect class I expression. 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.

Further studies on the mechanism of Tat-mediated repression have demonstrated that a class I promoter construct, consisting of only canonical promoter elements(68 bp fragment), is sensitive to repression. Thus, Tat-mediated repression appears to act directly on the preinitiation complex. Binding analyses indicate that Tat is able to bind promoter-binding complexes directly. However, the ability of Tat to repress the promoter is determined by the presence of upstream regulatory elements. Introduction of strong enhancers (i.e. viral enhancers) upstream of the promoter negates Tat repression. Characterization of the relevant enhancer elements is in progress.

Tat activation of the HIV LTR has been well characterized and functional Tat domains identified. A series of Tat mutants was constructed and tested for its ability both to repress class I and activate the viral LTR. Repression domains were found to be independent of activation requirements. Furthermore, a segment in the second exon has been mapped as necessary for the repression.

Publications:

None

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

PROJECT NUMBER
Z01 CB 09402-02 EIB

PERIOD COVERED
October 1, 1993 to September 30, 1994

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:	R. Grady	Biotechnology Fellow	EIB, NCI

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M. Clerici, Universita degli Studi, Milano, Italy

LAB/BRANCH
Experimental Immunology Branch

SECTION
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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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☐ (a) Human ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors B,A,D
☐ (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 (PBMC) from 47 patients newly diagnosed with Hodgkins disease (HD), As well as from 10 patients 10 patients with prostate cancer mononuclear cells. The PBL were stimulated in vitro with recall antigens (REC), HLA alloantigens (ALLO) and phytohemagglutinin (PHA). Four distinct patterns of Th responsiveness were detected in the HD patients: patients who responded to all three stimuli; patients who did not respond to REC; patients who responded only to PHA; and patients whose PBMC were refractory to all three stimuli. The more severely immune-compromised patients exhibited more severe hematologic parameters of HD. Of the 10 prostate cancer patients, four responded to all stimuli, five were unresponsive to REC, and one was unresponsive to REC and ALLO. These findings suggest that defects in cell mediated immunity exist in patients with certain types of cancer.

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. Forty-seven 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 laboratory has also initiated similar studies of Th immune dysfunction in patients with untreated prostate cancer, transitional cell carcinoma (bladder cancer) and renal cell carcinoma. Thus far, 10 patients each have been tested for prostate and renal cell cancers, and four have been tested for transitional cell tumors. PBMC from four of the prostate cancer patients responded to REC, ALLO, and PHA; PBMC from five failed to respond to REC, and one failed to respond to REC and ALLO. All PBMC from all 10 renal cancer patients responded to all stimuli, and three of the four transitional cell patients responded as healthy controls and one failed to respond to REC.

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 OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1993 through September 30, 1994

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) Molecular analysis of transacting 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 and acquired immunodeficiency diseases and cancer. 3) Genetic control of the immune response. One emphasis of this area is the definition of mechanisms of antigen presentation and T-lymphocyte recognition and the applications of these insights 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 for the treatment of leukemia/lymphoma and graft-versus-host-disease. 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 patients with primary and acquired immunodeficiency diseases, and 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.

REGULATION OF GENE EXPRESSION IN NORMAL AND MALIGNANT HUMAN LYMPHOCYTES

Dr. Lou Staudt's laboratory focuses on genes which regulate the development and function of normal human B lymphocytes and on genes which deregulate this program and cause lymphoid malignancies. Subtractive hybridization cDNA libraries enriched for genes expressed specifically in human B cell lymphomas were used in conjunction with automated DNA sequencing to isolate novel human lymphoid-restricted genes. 1305 subtracted cDNAs were sequenced and one half of the sequences were from novel human genes. Approximately 9% of the sequences encoded novel proteins that showed impressive amino acid sequence similarity to previously cloned genes. Northern blot analysis was used to confirm the identification of over 30 novel human lymphoid-restricted genes.

Three of these novel lymphoid-restricted genes which encode nuclear factors were selected for intensive analysis. The first gene, LAF-4, is homologous to AF-4, a gene which is involved in the recurrent t(4,11) translocation found in pre-B cell acute lymphocytic leukemia (ALL). Functional studies of LAF-4 and AF-4 demonstrated that they define a novel transcription factor family. The second gene encodes a zinc finger transcription factor which has been shown by several groups to be translocated in at least 20-30% of all cases of diffuse large cell lymphoma and has been termed BCL-6. The third gene, LySP100, is homologous to a nuclear autoantigen, SP100, which is localized to 5-15 discrete dots in the nucleus, termed "nuclear bodies". Although the function of nuclear bodies is unknown, it is intriguing that the product of the PML gene, which is involved in a characteristic t(15,17) translocation of acute promyelocytic leukemia, is also localized to this subnuclear organelle.

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 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.

Dr. Blaese's laboratory has also developed an 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.

MECHANISMS OF ANTIGEN-PRESENTATION AND T-LYMPHOCYTE RECOGNITION: APPLICATION TO VACCINE DESIGN

Dr. Berzofsky 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 found in murine studies that peptide vaccines for HIV can be made more potent or more broadly effective by selective introduction of mutations that improve binding to MHC or T-cell receptors. He and his associates developed methods to look for optimal sequences. To apply these approaches to human vaccines, he has been studying the binding of HIV envelope and reverse transcriptase peptides to human HLA molecules, and has raised HIV peptide-specific human CTL from peripheral blood of an uninfected individual. Dr. Berzofsky is studying escape mutations from CTL, and the effect of glycosylation of T-cell recognition. He developed synthetic vaccines for HIV that have broadly reactive HIV helper epitopes combined with CTL and neutralizing antibody epitopes, and has shown the importance of covalent linkage of helper and CTL epitopes on the same molecule for induction of CTL, never before demonstrated. These vaccine constructs were prepared for human phase I immunotherapy trials. The toxicology studies on these vaccine peptides were completed and were satisfactory. Dr. Berzofsky demonstrated the profound effect of cytokine imbalance on CTL activity and viral clearance in an animal model, and the ability to overcome the downregulation of human helper T cell responses in the blood of HIV infected individuals in vitro by use of another cytokine, IL-12. He identified two CTL epitopes in proteins of the hepatitis C virus, that causes liver cancer, using a novel approach, and is studying T cell responses to papillomavirus in patients with cervical dysplasia or cancer. He developed peptide cancer vaccines inducing CTL immunity to mutant p53 expressed in cancer cells, and showed in animal models some degree of protection against a tumor. In this system, he has shown the usefulness of interferon-gamma in sensitizing tumor cells for lysis by CTL. The first human patient has been treated in a phase I/II clinical trial of this mutant p53 or ras peptide vaccine approach to treating cancer.

THE MULTICHAIN IL-2 RECEPTOR: MOLECULAR CHARACTERIZATION AND USE AS A TARGET FOR IMMUNOTHERAPY

Dr. Waldmann's studies have focused on the role played by the IL-2/IL-2R system in normal and abnormal T-cell function and the use of these insights to develop IL-2R directed

therapy for leukemia/lymphoma. The IL-2R involves three IL-2 binding subunits, 55 kDa (IL-2Rα), 70-75 kDa (IL-2Rβ) and 64 kDa (IL-2Rγ). In a recent development Dr. Waldmann has defined a 15 kDa lymphokine he terms IL-T that stimulates T-cell proliferation and the activation of large granular lymphocytes. This lymphokine is distinct from lymphokines such as IL-2, IL-4, IL-7, IL-9 and IL-13 which act on T cells. By using an IL-3 dependent cell line prior to and following transfection with a gene encoding IL-2Rβ, Dr. Waldmann demonstrated that IL-T requires IL-2Rβ and IL-2Rγ expression for its action. One of Dr. Waldmann's most crucial contributions was his recognition that the IL-2R represents an extraordinarily useful therapeutic target. For example, Dr. Waldmann demonstrated that resting cells do not express IL-2Rα whereas a large number of IL-2Rα are expressed by malignant cells in adult T-cell leukemia (ATL), a disease associated with the expression of HTLV-I. Drs. Waldmann, Nelson and White completed a clinical trial with ^{90}Y-anti-Tac for patients with HTLV-I-associated ATL. Twelve of the 18 patients in this trial manifested a partial or complete remission following ^{90}Y-anti-Tac therapy. Recently Drs. Waldmann, White and coworkers extended these studies by initiating new clinical trials using ^{90}Y linked to humanized rather than murine anti-Tac to provide a relatively non-immunogenic agent for the treatment of an extended array of human leukemias and lymphomas. Furthermore, using a tumor model in nude mice Dr. Waldmann demonstrated the efficacy of the α-particle-emitting radiolabeled murine anti-Her-2/neu monoclonal antibody (^{212}Pb-AE1) in the prevention of development of human ovarian SK-OV-3 tumors that express Her-2/neu receptors. Thus the new insights concerning receptors on malignant cells taken in conjunction with the ability to produce humanized antibodies armed with radionuclides is providing a novel perspective for the treatment of certain neoplastic diseases.

GENETIC DEFECTS IN PRIMARY IMMUNODEFICIENCY DISEASES

Investigation by Dr. Nelson of the expression of the tyrosine kinase responsible for Bruton's X-linked agammaglobulinemia (XLA), BTK, in patients with X-linked agammaglobulinemia and isolated growth hormone deficiency has revealed normal abundance of full-length BTK mRNA. Sequence analysis of reverse transcribed and PCR amplified mRNA has revealed a benign polymorphism (T to C) at nucleotide 1899 of the coding sequence which does not change the encoded amino acid. These studies clearly distinguish XLA with growth hormone deficiency from Bruton's XLA at the molecular genetic level indicating that it is a distinct primary immunodeficiency disease and support the existence of additional X-chromosome genes that are critical in human B-cell development.

INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS

To test the hypothesis that endogenous IGF-II can modulate the trafficking of lysosomal enzymes by blocking binding to the IGF-II/mannose 6-phosphate receptor both intracellularly and at the cell surface, Dr. Nissley and coworkers transfected MCF-7 human breast cancer cells with two IGF-II containing vectors. One IGF-II construct represents the mature form of IGF-II and the other encodes a larger precursor form of IGF-II. MCF-7 cells were chosen because they produce large quantities of a particular lysosomal enzyme, cathepsin D. The

distribution of cathepsin D between the intracellular and extracellular compartments was examined in cloned cells transfected with vector alone and the two IGF-II vectors. Two protocols were employed. The first was a short metabolic labeling protocol with mannose 6-phosphate present in the medium to block uptake of lysosomal enzymes. Cathepsin D antiserum was used to immunoprecipitate cathepsin D from the media and cell extracts and the immunoprecipitates were analyzed by SDS/PAGE with quantitation with a PhosphorImager. The second protocol examined media and cell extracts by immunoblotting after a 48 hr incubation. Results from the first protocol, examining three clones each of vector only, mature IGF-II, and precursor IGF-II transfected cells, showed that the ratios of intracellular cathepsin D to extracellular cathepsin D were significantly lower in the cells transfected with the gene for precursor IGF-II compared to the cells transfected with vector only. These results support the hypothesis that intracellular IGF-II can block the targeting of lysosomal enzymes to lysosomes, resulting in increased secretion into the media.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CD-04002-25 MET

PERIOD COVERED

October 1, 1993 through September 30, 1994

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
Abdelkrim Alileche, M.D., Ph.D.	Fogarty Visiting Fellow	MET, NCI
Jack D. Burton, M.D.	Expert	MET, NCI
Don Eicher, M.D.	Clinical Associate	MET, NCI
Kayhan Garmestani, Ph.D.	Visiting Scientist	MET, NCI
Carolyn K. Goldman	Microbiologist	MET, NCI
Terry Jackson-White, M.D.	Biotechnology Fellow	MET, NCI
Claude Kasten-Sportès, M.D.	Senior Staff Fellow	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:	PROFESSIONAL:	OTHER:	
13	11	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's studies have focused on the role played by the IL-2/IL-2R system in normal and abnormal T-cell function and the use of these insights to develop IL-2R directed therapy for leukemia/lymphoma. The IL-2R involves three IL-2 binding subunits, 55 kDa (IL-2Rα), 70-75 kDa (IL-2Rβ) and 64 kDa (IL-2Rγ). In a recent development Dr. Waldmann has defined a 15 kDa lymphokine he terms IL-T that stimulates T-cell proliferation and the activation of large granular lymphocytes. This lymphokine is distinct from lymphokines such as IL-2, IL-4, IL-7, IL-9 and IL-13 which act on T cells. By using an IL-3 dependent cell line prior to and following transfection with a gene encoding IL-2Rβ, Dr. Waldmann demonstrated that IL-T requires IL-2Rβ and IL-2Rγ expression for its action. One of Dr. Waldmann's most crucial contributions was his recognition that the IL-2R represents an extraordinarily useful therapeutic target. For example, Dr. Waldmann demonstrated that resting cells do not express IL-2Rα whereas a large number of IL-2Rα are expressed by malignant cells in adult T-cell leukemia (ATL), a disease associated with the expression of HTLV-I. Dr. Waldmann has completed a clinical trial with ^{90}Y-anti-Tac for patients with HTLV-I-associated ATL. Twelve of the 18 patients in this trial manifested a partial or complete remission following ^{90}Y-anti-Tac therapy. Recently Dr. Waldmann has extended these studies by initiating new clinical trials using ^{90}Y linked to humanized rather than murine anti-Tac to provide a relatively non-immunogenic agent for the treatment of an extended array of human leukemias and lymphomas. Furthermore, using a tumor model in nude mice Dr. Waldmann demonstrated the efficacy of the α-particle-emitting radiolabeled murine anti-Her-2/neu monoclonal antibody (^{212}Pb-AE1) in the prevention of development of human ovarian SK-OV-3 tumors that express Her-2/neu receptors. Thus the new insights concerning receptors on malignant cells taken in conjunction with the ability to produce humanized antibodies armed with radionuclides is providing a novel 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
Gloria Kurys Szakiel, Ph.D.	IRTA Fellow	MET, NCI

Project Description

Major Findings:

Dr. Waldmann's studies have focused on the role played by the IL-2/IL-2 receptor (IL-2R) system in normal and abnormal T-cell activation, proliferation and function and the use of these insights to develop IL-2R-directed therapy for leukemia/lymphoma. His investigations have led to the definition and biochemical characterization of the p55 (IL-2Rα) and p70-75 (IL-2Rβ) IL-2 binding proteins that participate in the high affinity IL-2R. Recently Sugamura and coworkers have defined a third p64 (IL-2Rγ) IL-2 binding protein that cooperates with the other two subunits in the formation of the high affinity IL-2R. In a recent development Dr. Waldmann has defined a 14 kDa lymphokine he terms IL-T that stimulates T-cell proliferation and the activation of large granular lymphocytes. This lymphokine is distinct from lymphokines such as IL-2, IL-4, IL-7, IL-9 and IL-13 which act on T cells. The addition of this cytokine to a lymphokine-dependent T-cell line yields a stimulation index of over 250. In addition, by using an IL-3 dependent cell line prior to and following transfection with a gene encoding IL-2Rβ, Dr. Waldmann demonstrated that IL-T requires IL-2Rβ and IL-2Rγ expression for its action. This observation may explain the paradox that mice with a deletion of the IL-2 gene by homologous recombination manifest only modest immunodeficiency whereas disorders of IL-2Rγ that lead to X-linked severe combined immunodeficiency disease (SCID) cause profound defects of both T- and B-cell function.

One of Dr. Waldmann's most crucial contributions was his recognition that the IL-2R represents an extraordinarily useful therapeutic target. He entered this area to exploit his observation that in contrast to the lack of Tac-protein expression in normal resting mononuclear cells this receptor is expressed by a proportion of the abnormal cells in certain forms of lymphoid neoplasia [e.g., cutaneous T-cell lymphomas (CTCL), hairy cell leukemia and Hodgkin's disease], in select autoimmune disorders and by the T-cells of individuals rejecting allografts. For example, Dr. Waldmann demonstrated that an unusually large number of IL-2Rs are expressed by malignant cells in ATL a disease which is associated with the expression of the HTLV-I encoded transactivation gene product, *tax*. He reasoned that agents which could eliminate Tac-expressing leukemic cells as well as activated T-cells involved in other disease states might provide effective therapy for these disorders. Dr. Waldmann and his collaborators developed a wide variety of agents that are directed toward eliminating IL-2R-expressing cells. In the initial studies Dr. Waldmann performed a trial of intravenously administered anti-Tac for the treatment of patients with ATL. ATL is an aggressive leukemia with no standard curative therapy that had a median survival duration of 9 months. The patients did not suffer untoward reactions following anti-Tac therapy. Seven of the 19 patients studied had a transient mixed (1), partial (4) or complete remission (2) lasting from one to over 48 months following therapy. Recently to circumvent the problems inherent in the use of murine monoclonal antibodies, Dr. Waldmann in conjunction with Cary Queen using genetic engineering produced humanized anti-Tac antibodies by combining the complementarity-determining regions of murine anti-Tac with human IgG1, kappa framework and constant regions. Dr. Waldmann extended the clinical therapeutic implications of monoclonal antibodies by focusing on the use of these agents as carriers of cytotoxic agents. In pivotal studies Dr. Waldmann and his collaborator Otto Gansow developed alternative cytotoxic agents wherein α- and β-emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. Dr. Waldmann has just completed a clinical trial with ^{90}Y-anti-Tac for patients with HTLV-I-associated ATL. Twelve of the 18 patients in this trial manifested a partial or complete remission following ^{90}Y-anti-Tac therapy that

has been sustained in some patients for over 36 months of observation. Most recently Dr. Waldmann extended these studies by initiating new clinical trials using ^{90}Y linked to humanized rather than murine anti-Tac to provide a relatively non-immunogenic agent for the treatment of an array of human leukemias and lymphomas.

A centerpiece of Dr. Waldmann's present efforts involves the use of humanized monoclonal antibodies armed with the α-emitting radionuclides ^{212}Bi, and ^{212}Pb that release high energy emissions over a short distance thus maintaining the specificity of the monoclonal antibody action. Dr. Waldmann showed that ^{212}Bi, an α-emitting radionuclide conjugated to anti-Tac by use of a bifunctional chelate, is well suited for select forms of immunotherapy. In a murine therapy model involving murine tumors transfected with the gene encoding the human Tac peptide, Dr. Waldmann showed that ^{212}Bi anti-Tac provides effective therapy for this malignancy when used in the adjuvant setting. Thus this radionuclide may be useful in select situations such as adjuvant or intracavitary therapy, strategies that target the vascular endothelial cells of tumors or in the treatment of leukemias. In an extension of these studies using a tumor model in nude mice Dr. Waldmann and associates evaluated the efficacy of the α-particle-emitting radiolabeled murine anti-Her-2/neu monoclonal antibody (^{212}Pb-AE1) in the prevention of development of human ovarian SK-OV-3 tumors that express Her-2/neu receptors. Tumor development was prevented in 100% of animals that were injected with SK-OV-3 cells subcutaneously when treated three days subsequently with ^{212}Pb-AE-1. This efficacy in the adjuvant setting was antibody-specific since treatments with equally labeled irrelevant monoclonal antibody or unlabeled AE-1 were not effective. Thus the anti-Her-2/neu monoclonal antibody armed with the α-emitting radionuclide ^{212}Pb may be effective in the therapy of micrometastases of Her-2/neu-expressing ovarian, breast and gastric tumors. In summary, the new insights concerning receptors on human malignant cells taken in conjunction with the ability to produce humanized antibodies armed with radionuclides are providing a novel perspective for the treatment of certain neoplastic diseases.

Honors:

1993 Honor Lecture, The First Annual Joseph Goldberger Clinical Investigator Lecture, NIH
1994 Albert Coons Memorial Lecture, Harvard Medical School
1994 Jules Bordet Lectureship, New York Academy of Science

Publications:

Anasetti C, Hansen JA, Waldmann TA, Binger M-H, Hakimi J, Light SE, Mould D, Satoh H, Appelbaum FR, Davis J, Deeg HJ, Doney K, Martin PJ, Nash R, Storb R, Sullivan KM, Witherspoon RP. Treatment of acute graft-versus host disease with H-anti-Tac: a humanized antibody that binds to the interleukin-2 receptor. Blood. In press.

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Tiffany LJ, Dobbs D, Garmestani K, Raubitschek A, Tinubu A, Brechbiel MW, Goffman T, Gansow OA, Waldmann TA, Kozak RW. Radiolabeled CHX_B-2E4 is stable in vivo and is a suitable immunoconjugate for testing α-particle-emitting bismuth radionuclides in interleukin-2 receptor targeted immunotherapy. Cancer Res. In press.

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Waldmann TA, White JD, Goldman CK, Top L, Grant A, Bamford R, Roessler E, Horak I, Zaknoen S, Kasten-Sportès C, England R, Horak E, Mishra B, Dipre M, Hale P, Fleisher TA, Junghans RP, Jaffe ES, Nelson DL. The interleukin-2 receptor: a target for anti-Tac monoclonal antibody therapy of human T-cell lymphotropic virus I-induced adult T-cell leukemia. Blood 1993;82:1701-12.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04015-5 MET

PERIOD COVERED
October 1, 1993 through September 30, 1994

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
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 Leichtling, Ph.D.	Senior Staff Fellow	MET, NCI
Craig Mullen, M.D., Ph.D.	Commissioned Officer	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:	PROFESSIONAL:	OTHER:
11	8	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.)*

Michael Blaese's laboratory continues to focus on the development of gene therapy. He led the group which performed the first successful 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

Jay Ramsey, M.D., Ph.D.	Clinical Associate	MET, NCI
Renaud Touraine, M.D.	Special Volunteer	MET, NCI

Project Description

Major 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. 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. 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, 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), 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 β_2 microglobulin. This defect is corrected by the addition of exogenous β_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 1994:

Naomi M. Kanof Clinical Investigator Award - The Society for Investigative Dermatology
20th Wolf-Zuelzer Memorial Lecture, Wayne State University School of Medicine
Inaugural Keynote Lecture, Verbund Klinisch-Biomedizinische Forschung, dkfz, Heidelberg, Germany
Keynote Address, 23rd Annual Scientific-Educational Meeting of the Society for Critical Care Medicine
Robert T. Wong Lectureship, University of Hawaii School of Medicine
9th Colleen Giblin Memorial Lectureship, Columbia University College of Medicine

Publications:

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Ram Z, Walbridge S, Oshiro EM, Viola JJ, Chaing Y,Mueller SN, Blaese RM, Oldfield EH. Intrathecal gene therapy for the treatment of meningeal carcinomatosis. Cancer Res, in press.

Rosenberg SA, Anderson WF, Blaese RM, Hwu P, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Ettinghausen SE, Parkinson DN, White DE. The development of gene therapy for the treatment of cancer. Ann Surg 1993;218:455-64.

Walker R, Blaese RM, Carter CS, Chang L, Klein H, Lane HC, Leitman SF, Mullen CA, Larson M. A study of the safety and survival of the adoptive transfer of genetically marked syngeneic lymphocytes in HIV-infected identical twins. Hum Gene Ther 1993;4:659-80.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04016-21 MET

PERIOD COVERED
October 1, 1993 through September 30, 1994

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
Bhakta Dey, Ph.D.	Fogarty Visiting Fellow	MET, NCI
Richard Furlanetto, M.D., Ph.D.	Sabbatical (U Rochester)	MET, NCI
Barbara Jud, M.D.	Research 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:	PROFESSIONAL:	OTHER:
5.6	4.6	1.0 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.)*

To test the hypothesis that endogenous IGF-II can modulate the trafficking of lysosomal enzymes by blocking binding to the IGF-II/mannose 6-phosphate receptor both intracellularly and at the cell surface, we have transfected MCF-7 human breast cancer cells with two IGF-II containing vectors. One IGF-II construct represents the mature form of IGF-II and the other encodes a larger precursor form of IGF-II. MCF-7 cells were chosen because they produce large quantities of a particular lysosomal enzyme, cathepsin D. The distribution of cathepsin D between the intracellular and extracellular compartments was examined in cloned cells transfected with vector alone and the two IGF-II vectors. Two protocols were employed. The first was a short metabolic labeling protocol with mannose 6-phosphate present in the medium to block uptake of lysosomal enzymes. Cathepsin D antiserum was used to immunoprecipitate cathepsin D from the media and cell extracts and the immunoprecipitates were analyzed by SDS/PAGE with quantitation with a PhosphorImager. The second protocol examined media and cell extracts by immunoblotting after a 48 hr incubation. Results from the first protocol, examining three clones each of vector only, mature IGF-II, and precursor IGF-II transfected cells, showed that the ratios of intracellular cathepsin D to extracellular cathepsin D were significantly lower in the cells transfected with the gene for precursor IGF-II compared to the cells transfected with vector only. These results support the hypothesis that intracellular IGF-II can block the targeting of lysosomal enzymes to lysosomes, resulting in increased secretion into the media. Experiments utilizing the second protocol are in progress.

The cytoplasmic domain of the IGF-I receptor containing the receptor tyrosine kinase has been expressed in a baculovirus system and purification of the expressed protein is in progress.

Project Description

Major Findings:

Cathepsin D trafficking in MCF-7 human breast cancer cells overexpressing insulin-like growth factor II.

Shortly after it was demonstrated that the IGF-II receptor is bifunctional, binding a growth factor and targeting lysomal enzymes to lysosomes, we reported that IGF-II inhibited the uptake of the lysosomal enzyme, β-galactosidase, by two cell lines in monolayer culture. We also demonstrated that IGF-II blocked the binding of ^{125}I-β-galactosidase to pure IGF-II/M6P receptor in solution. We predicted that a cell that was producing IGF-II would have increased concentrations of lysosomal enzymes in conditioned media. We also speculated that the inhibition of lysosomal enzyme binding to the IGF-II/M6P receptor might be occurring intracellularly such as in the Golgi cisternae, leading to a decrease in the flux of lysosomal enzymes to lysosomes and an increase in secretion which is viewed as a default pathway. To test this hypothesis we have transfected MCF-7 human breast cancer cells with two IGF-II expression vectors, one coding for mature M_r 7471 IGF-II containing B,C,A, and D domains, and a precursor form containing B,C,A,D, and E domains. The precursor form of IGF-II was included because it might be more effective in inhibiting the binding of lysosomal enzymes if inhibition is by steric hindrance, and because tumor cells produce predominantly precursor IGF-II rather than mature IGF-II. MCF-7 cells were chosen because breast cancer cell lines have been observed to produce large quantities of a particular lysosomal enzyme, cathepsin D.

Mature and precursor IGF-II cDNA products were produced by PCR methodology 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 MCF-7 cells were transfected with the two IGF-II expression vectors or with vector alone and the cells were selected in Genetisin. Conditioned media from the surviving mass cultures of cells transfected with the two IGF-II vectors were shown to be producing the mature and precursor form of IGF-II using immunoblotting with a monoclonal antibody to IGF-II. Vector and IGF-II transfectants were cloned by limiting dilution and screened for IGF-II production by a sensitive dot-blot assay based on Amersham ECL methodology. For unknown reasons, all of the clones of the MCF-7 cells producing the precursor form of IGF-II secreted much higher levels of IGF-II (as high as 10 ug/ml) than did the cells transfected with the mature IGF-II vector construct.

The distribution of cathepsin D between the intracellular and extracellular compartments in the vector and IGF-II transfected cells was assessed by immunoprecipitation and immunoblotting experiments using antisera specific for cathepsin D. These antisera were raised in rabbits using as antigen cathepsin D which we purified from human placenta. We first employed a metabolic labeling protocol with a 1 hr labeling period with ^{35}S labeled methionine/cystine followed by a 4 hr chase. The medium contained 10 mM mannose 6-phosphate, sufficient to block uptake of lysosomal enzymes from the medium via the IGF-II/M6P receptor. Media and cell extract samples were immunoprecipitated with cathepsin D antiserum and Protein A-Sepharose. The immunoprecipitates were analyzed by SDS/polyacrylamide gel electrophoresis and autoradiography. The radioactivity in the cathepsin D bands was quantitated with a PhosphorImager and a ratio was calculated between the intracellular, mature form (30 kDa) and the extracellular, precursor form (50 kDa) of the enzyme. In the first series of experiments which examined three clones each of vector only, mature IGF-II, and precursor IGF-II transfected cells, the ratios of intracellular to extracellular cathepsin D were significantly lower ($p < 0.05$) in the cells transfected with the precursor form of IGF-II compared to

the vector transfected cells. Since mannose 6-phosphate is present in the media during the metabolic labeling, this protocol examined the question whether or not endogenous IGF-II modulates the intracellular pathway for handling of newly synthesized lysosomal enzymes. The results are consistent with the prediction that intracellular IGF-II can block the delivery to lysosomal enzymes to lysosomes, resulting in an altered distribution between the intracellular and extracellular compartments.

In a second experimental protocol, we are examining the distribution of cathepsin D between the intracellular and extracellular compartments after a 48 hr incubation. Cathepsin D is measured in the media and cell extract by immunoblotting with cathepsin D antiserum, using ^{125}I-Protein A to label the cathepsin D bands on the immunoblot. The PhosphorImager is used to quantitate the radioactivity in the cathepsin D bands. Preliminary experiments have demonstrated the feasibility of this approach and suggest that the levels of cathepsin D in the media are much higher in the cells transfected with the IGF-II vectors. Since mannose 6-phosphate is not present in the media during these experiments, the distribution of cathepsin D between the intracellular and extracellular compartments would be expected to be a summation of the action of IGF-II blocking cathepsin D uptake from the media and blocking cathepsin D traffic to lysosomes intracellularly.

Cathepsin D levels in breast cancer tissue have been shown to be positively correlated with the likelihood of nodal spread of the malignancy. Many tumor cells in culture have been shown to produce IGF-II. Our results show that IGF-II production increases the extracellular concentration of a particular lysomal enzyme, cathepsin D. Perhaps extracellular cathepsin D (and other lysosomal enzymes) could play a role in tumor spread by digesting matrix?

Expression of the cytoplasmic domain of the IGF-I receptor in insect cells.

The insulin receptor and the IGF-I receptor are closely related and belong to the family of receptors with intrinsic tyrosine kinase activity. The cytoplasmic domain of the insulin receptor has been expressed by others in insect cells using a baculovirus system and the purified cytoplasmic domain was found to be fully active as a tyrosine kinase, leading to the conclusion that the native receptor is in a constrained conformation and insulin binding simply releases the tyrosine kinase from this inhibited state. We decided to attempt to express the cytoplasmic domain of the IGF-I receptor in insect cells using a baculovirus system with several goals in mind. We want to confirm the expectation that like the insulin receptor, the cytoplasmic domain of the IGF-I receptor will be fully active as a kinase upon the addition of ATP. If so, the receptor will be in an active conformation and would be expected to bind to downstream effectors in the signaling pathway. It can therefore be used as a reagent in affinity chromatography to bind components from cell extracts from IGF-I responsive cells and following ^{32}P labeling it could also be used to screen expression libraries. To these ends we have made an IGF-I receptor cytoplasmic domain cDNA using PCR methodology and inserted this construct into the pBlueBacHis vector. Recombinants with wild type baculovirus were selected and used to infect High 5 insect cells. Rabbit antisera raised against a peptide in the cytoplasmic domain of the IGF-I receptor have been utilized to demonstrate that a polypeptide of the expected size is being produced by the insect cells. Purification will now be attempted using a nickel affinity column.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04017-16 MET

PERIOD COVERED
October 1, 1993 through September 30, 1994

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
S. Bhagovati, M.D.	Expert	MET, NCI
Donn Stewart, M.D.	Clinical Associate	MET, NCI
Stephanie Trieber-Held, M.D.	Visiting Fellow	MET, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Metabolism Branch

SECTION
Immunophysiology

INSTITUTE AND LOCATION
DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	4	0.8 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.)*

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. 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. Investigation of the expression of the tyrosine kinase responsible for Bruton's X-linked agammaglobulinemia (XLA), BTK, in patients with X-linked agammaglobulinemia and isolated growth hormone deficiency has revealed normal abundance of full-length BTK mRNA. Sequence analysis of reverse transcribed and PCR amplified mRNA has revealed a benign polymorphism (T to C) at nucleotide 1899 of the coding sequence which does not change the encoded amino acid. These studies clearly distinguish XLA/GHD from Bruton's XLA at the molecular genetic level and support the existence of additional X-chromosome genes that are critical in human B-cell development.

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 α (55 kDa) and β (75 kDa) chains of the IL-2 receptor (IL-2R). Using hybridoma-derived monoclonal antibodies to the IL-2Rα, we have identified a soluble form of this molecule which is 10 kDa smaller than the cell surface form of IL-2Rα and established an Enzyme-Linked ImmunoAssay (ELISA) for the measurement of this molecule in serum.

Elevated levels of soluble IL-2Rα 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-2Rα were also observed in allograft rejection episodes and exacerbations in autoimmune diseases. Reductions in sIL-2Rα correlated with responses to therapy in patients with ATL and HCL. The measurement of sIL-2Rα 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). We have compared patients from the original family with XLA/GHD and those with Bruton's agammaglobulinemia (XLA/GHD). As determined by restriction fragment length polymorphism studies and multipoint linkage analysis, the odds are 250:1 against the XLA/GHD gene mapping to the XLA location. Studies of the randomness of X-chromosome inactivation in obligate XLA/GHD carriers revealed non-random X-chromosome inactivation in both T-cells and B-cells as opposed to B-cells only in XLA. A patient with XLA/GHD was shown to have normal abundance of full-length Bruton's tyrosine kinase (BTK) mRNA in peripheral blood mononuclear cells by Northern analysis. Finally, automated sequencing of reverse transcribed and polymerase chain reaction amplified mRNA from the XLA/GHD patient revealed a single base pair benign polymorphism (T to C) at nucleotide 1899 of the coding sequence which did not change the encoded amino acid. These studies clearly distinguish XLA/GHD from Bruton's XLA at the molecular genetic level and support the existence of additional X-chromosome genes that are critical in human B-cell development. 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 have cloned full-length cDNAs for BTK and have generated retroviral gene constructs. We 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:

Waldmann TA, White J, Gansow O, Kasten-Sportes C, Roessler E, Zaknoen S, Horak I, Carrasquillo JA, Reynolds JC, Junghans R, England R, Goldman C, Bamford R, Horak E, Tinubu SA, Top L, Dipre M, Mishra B, Grant A, Nelson D. Treatment of malignant skin diseases with genetically engineered molecules armed with radionuclides. In: Burgdorf WHC, Katz SI. Dermatology Progress and Perspectives: Proceedings of the 18th World Congress of Dermatology. New York: Parthenon, 1993;61-63.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04020-17 MET

PERIOD COVERED

October 1, 1993 through September 30, 1994

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
Martha Alexander-Miller, Ph.D.	IRTA Fellow	MET, NCI
Richard England, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
Kazutak Kurokohchi, M.D., Ph.D.	Visiting Fellow	MET, NCI
Graham Leggett, Ph.D.	Visiting Fellow	MET, NCI
Margaret Marshall, M.D.	Clinical Associate	MET, NCI
M. Charles Smith, M.D., Ph.D.	Clinical Associate	MET, NCI
Taku Tsukui, M.D., Ph.D.	Postdoctoral IRTA	MET, NCI

COOPERATING UNITS *(if any)*

Gene M. Shearer, Ph.D.	Section Chief	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:	PROFESSIONAL:	OTHER:
11	9	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.)*

We 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. We have found in murine studies that peptide vaccines for HIV can be made more potent or more broadly effective by selective introduction of mutations that improve binding to MHC or T-cell receptors. We have developed methods to look for optimal sequences. To apply these approaches to human vaccines, we have been studying the binding of HIV envelope and reverse transcriptase peptides to human HLA molecules, and have raised HIV peptide-specific human CTL from peripheral blood of an uninfected individual. We are studying escape mutations from CTL, and the effect of glycosylation of T-cell recognition. We have developed synthetic vaccines for HIV that have broadly reactive HIV helper epitopes combined with CTL and neutralizing antibody epitopes, and have shown the importance of covalent linkage of helper and CTL epitopes on the same molecule for induction of CTL, never before demonstrated. We have prepared these vaccine constructs for human phase I immunotherapy trials. The toxicology studies on these vaccine peptides were completed and were satisfactory, and the protocol was approved with minor revisions by the IRB. The IND is being prepared for the FDA. We have shown the profound effect of cytokine imbalance on CTL activity and viral clearance in an animal model, and the ability to overcome the downregulation of human helper T cell responses in the blood of HIV infected individuals in vitro by use of another cytokine, IL-12. We have identified two CTL epitopes in proteins of the hepatitis C virus, that causes liver cancer, using a novel approach, and are studying T cell responses to papillomavirus in patients with cervical dysplasia or cancer. We have developed peptide cancer vaccines inducing CTL immunity to mutant p53 expressed in cancer cells, and have shown in animal models some degree of protection against a tumor. In this system, we have shown the usefulness of interferon-gamma in sensitizing tumor cells for lysis by CTL. Our first human patient has been treated in a phase I/II clinical trial of this mutant p53 or ras peptide vaccine approach to treating cancer.

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
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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
Stephen Hoffman, M.D.	Commander	ID, NMRI
Mark Schiffman, M.D.	Senior Investigator	EEB, DCE, NCI
John Caligan, Ph.D.	Lab Chief	NIAID
Paul Klotman, M.D.	Senior Investigator	NIDR
Michael Mage, Ph.D.	Senior Investigator	NCI
Robert Yarchoan, M.D.	Senior Investigator	MB, NCI
Bruce Johnson, M.D.	Senior Investigator	NMOB, NCI

Project Description

Major Findings:

We have been studying the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, the factors that determine which antigenic structures are more likely to be recognized, and the application of these principles to the design of synthetic vaccines for AIDS, 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. As a model antigen, because of our interest in HIV vaccines, we have used the envelope protein of HIV, gp160. We had earlier found that two apparently unrelated epitopes of gp160 crossreacted for recognition by CD8 cytotoxic T lymphocytes (CTL), even though there was no sequence homology. We have now made 26 increasingly truncated peptides to map the minimal sites, and have found an 8-residue sequence in one and a 10-residue sequence in the other. The homology is still not apparent, but now we can model the structures of these minimal peptides to try to understand the crossreaction. Both of these peptides are components of vaccines we are developing (see below).

In a collaborative study with Dr. Hanah Margalit, formerly at NCI but now in Jerusalem, we have developed a way of identifying motifs for sequences in proteins that can bind to particular MHC molecules. We have developed a database of known T cell epitopes presented by different MHC molecules, and ones that are known not to bind to those molecules. We have developed an algorithm that compares peptides that are presented with those that are not and searches for sequence patterns shared by those that bind and absent in those that do not. Instead of searching for patterns of individual amino acids, we search for specific properties, such as hydrophobicity, hydrogen bonding capability, aromaticity, charge, size, etc. We have been able to find highly significant patterns. Of interest is the fact that negative attributes are as important as positive ones. This result supports

our findings from experimental studies reported last year indicating that adverse interactions are as important in determining whether a peptide will bind to a particular MHC molecule as are favorable interactions of anchor residues.

The above studies and studies reported last year indicated that it was possible to alter sequences of peptides to make them more potent vaccines in mice, by increasing their affinity for the murine MHC molecules without altering their aspect recognized by the T cell receptor. To extend this approach to human vaccines, we have had to carry out similar studies with HIV peptides presented by human HLA molecules. Peptide 18 (P18) of the V3 loop of gp160 was found in our earlier studies to be an immunodominant CTL epitope in mice, as well as a CTL epitope recognized by human CTL with at least three different human HLA molecules, HLA-A2, A3, and either A1 or B8. It is also a component of the vaccine we are developing (see below). We have therefore begun studying the binding of this peptide to HLA-A2 and A3, using both recombinant HLA molecules, with John Coligan's group, and also mutant cell lines that express HLA-A2 or A3 only when cultured with peptides that can bind to these. Initial results are interesting because they do not fit the now-classical motifs for sequences binding to these HLA molecules. Once we have defined the minimal peptides, we can begin studying mutations that might improve efficacy. It will also be necessary to have human T cells reactive with these peptides, to test activity. To this end, we have finally just succeeded in raising a human CTL line specific for P18 by primary in vitro stimulation of peripheral blood cells from an HIV-seronegative blood donor, in order to avoid working with infected cells. We are determining the genetic restriction and fine specificity of this line. These results will be invaluable for optimizing a peptide vaccine for human use.

Because this same P18 peptide is also presented by class II molecules to helper T cells, we have also been studying the binding to class II MHC molecules. Studies we reported last year showed that there was a remarkable concordance between residues involved in binding of this peptide to class I MHC D^d and to class II I-A^d molecules. We have now just identified a mutation that leads to greatly increased potency for stimulation of helper T cells, by at least two orders of magnitude. Thus, such improvements in efficacy by epitope engineering can be applied to helper T cells as well.

We are also trying to determine whether escape mutations will arise in this epitope of gp160 in a collaborative study with Prof. Satvir Tevethia at Hershey Medical Center, PA. Dr. Tevethia has placed this epitope in two positions in the SV40 T antigen gene and inserted this construct in a tumor cell line. We are now selecting escape mutants of this cell line using a murine CTL line specific for P18. Escape mutants can then be sequenced, and the effect of the substitutions on MHC binding and T-cell receptor binding can then be determined.

In collaboration with Dr. Michael Mage, NCI, we have been studying complexes of recombinant single chain H-2D^d-beta-2 microglobulin associated with the minimal 10-mer of P18, called I10. We have shown that these complexes can stimulate specific CTL in vitro. We are developing ways of immunizing directly with these complexes, as well as using them to derive antibodies specific for the peptide-MHC complex.

We are also studying the effect of glycosylation on T-cell recognition of this HIV epitope. Vaccinia constructs have been made by Dr. Peter Nara's group (NCI) that have mutations in and near this site that create N-glycosylation sites. We have prepared peptides corresponding to these mutations that will not be glycosylated because they are not synthesized in the cell. With these reagents, and glycosylation inhibitors, we can examine to what extent N-glycosylation interferes with antigen processing or T-cell recognition.

We have also recently mapped the murine minimal epitope of an HIV reverse transcriptase peptide that we had previously shown to be recognized by both human and murine CTL. We are now examining substitutions in this peptide for binding to H-2K^k and T-cell receptor recognition, and also trying to map the human HLA restriction. This peptide may be useful in future vaccines because it is more conserved than many of the envelope sequences.

To induce CTL with peptide vaccines, we have also shown that physical association between a helper epitope and a CTL epitope is necessary for induction of CTL. This association can be achieved by covalent linkage, as in our vaccine constructs discussed below, or by trapping the two together in microdroplets of an emulsion adjuvant. However, in the absence of physical trapping, the covalent linkage is necessary. This requirement is probably due to the need to have both epitopes presented by the same antigen presenting cell, so that the helper T cell can secrete cytokines in the local environment of the CTL that is being activated, and so that the helper T cell can upregulate costimulatory molecules on the antigen presenting cell that are necessary for CTL activation. Although such covalent hapten-carrier linkage was long known for antibody responses, it had never been demonstrated for CTL.

In the arena of HIV vaccines, we have focused on two synthetic peptide constructs containing the multideterminant cluster helper epitopes we described earlier 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 IIIB 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 define 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 $CD8^+$ CTL. We have had this peptide and the PCLUS3-18MN peptide made under GMP conditions for a phase I human trial for immunotherapy. These peptides have been formulated in a human grade of incomplete Freund's adjuvant, called Montanide ISA-51 from Seppic, Inc. Incomplete Freund's turned out to be the best of the adjuvants tested for all responses measured, neutralizing antibodies, CTL, and Th1 helper T-cell responses, as measured by production of IL-2 and not IL-4. This formulation has now just completed toxicology studies in rabbits that are necessary prior to obtaining an IND for the clinical trial. The rabbits produced a strong antibody response to both peptide constructs, but showed not toxicity, except for minor local irritation at the site of injection. The clinical protocol for an immunotherapy trial of these vaccine peptides, to be performed with Dr. Robert Yarchoan, NCI Medicine Branch, has been written and approved with minor revisions by the IRB and CTEP. The minor revisions have been made and resubmitted for approval. The next step is the filing of the IND with the FDA. The phase I immunotherapy trial would be to immunize asymptomatic HIV seropositive patients to determine whether they develop an increase in antibodies or CTL, or develop antibodies or CTL to epitopes not previously recognized. Safety would also be assessed, as well as any impact on the stability of their CD4 counts and other disease parameters.

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, such as HIV gp160-expressing vaccinia virus. We had previously 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 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 $CD3^+$ but $CD4^-8^-$ T cell, and is currently being characterized. It is also mediated by a soluble factor which can act across a semipermeable membrane. 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, with regard to HIV, 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 IL-12 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 IL-12 which upregulates Th1 responses and bypasses the effect of IL-10, in a therapeutic protocol.

With regard to hepatitis C, we have succeeded in identifying CTL epitopes in two proteins, the core and the NS3 protein. One epitope in a conserved region of the core protein is interesting in that it is seen by both murine CTL and human CTL, the latter with HLA-A2, the most common human class I MHC molecule, present in about 46% of the population. Thus, the peptide could be useful in a large fraction of the human population, and also can be studied experimentally in animals. Also, we have identified a new epitope in the NS3 protein of HCV by a novel approach of proteolytically digesting the recombinant protein, separating the fractions by HPLC, testing these for activity with human CTL, sequencing the active fractions, and then synthesizing the peptides to confirm that the peptides sequenced are responsible for the activity in the HPLC fraction. This approach could be useful for other proteins for which it is not feasible to make large numbers of overlapping synthetic peptides to map epitopes.

For human papillomavirus, we have screened large numbers of patients with cervical dysplasia for cytokine responses to overlapping peptides from HPV-16 E6 and E7 oncoproteins. A significant fraction of these patients have T cells that make IL-2 in response to mixtures of peptides, and we are now mapping individual epitopes. We are also screening for CTL activity, but the amount of blood available has been limiting. We have obtained recombinant vaccinia viruses expressing E6 and E7 from Dr. Bernard Moss, NIAID, that we can use to stimulate CTL or to make targets expressing the whole protein.

In the area of cancer vaccines, we have made additional progress, both in the murine studies and in the human clinical trial. In the murine tumor protection studies, we tried a number of experiments to see whether immunization with the synthetic peptide corresponding to the mutant p53 peptide T1272 would cause rejection of fibrosarcomas produced by murine fibroblast tumor lines cotransfected with the mutant p53 and ras. We learned that the tumor cells that expressed mutant ras as well as p53 were not good targets for CTL unless we grew them in interferon-gamma to upregulate the major histocompatibility (MHC) molecules and transporter proteins. This made the tumor cells better targets for CTL in vitro, but posed a dilemma in vivo. The ras-expressing tumor cells produced tumors even in the immunized mice whether or not they were grown in interferon-gamma. Probably the effect of interferon-gamma treatment before injection did not last long enough once the tumor cells were injected into the animal to allow rejection in vivo. We plan to test whether treatment of the mice with interferon-gamma after injection of the tumor will allow immunized mice to reject the tumor. Thus, although we learned that interferon-gamma can potentially overcome one mechanism by which tumors escape from immune recognition, it became apparent that the fibroblast tumors were not a good model for the in vivo protection studies.

The murine mastocytoma P815 has long been used as a target for CTL and expresses high levels of class I MHC molecules. Therefore, Frank Ciernick in Dave Carbone's lab transfected P815 cells with the T1272 mutant p53 gene, and we obtained our first promising in vivo results. Two types of transfectants were made, one expressing a minigene corresponding to the segment of p53 containing the mutation, and one expressing the whole mutant p53 protein. In preliminary experiments with small numbers of animals, 3 BALB/c mice immunized with the mutant T1272 peptide were completely protected against the T1272-minigene transfected P815 tumor, whereas the three unimmunized control mice all died within 37 days of receiving the tumor cells. In a second experiment with P815 tumors expressing the whole mutant p53 protein, 3 of 5 immunized mice survived compared to 1 of 5 unimmunized mice. However, the P815 tumor derives from DBA/2 mice, not BALB/c. Although these strains share the same MHC genes, they differ in minor histocompatibility antigens. Since the control unimmunized BALB/c mice died from the P815 tumors, we knew that the minor histocompatibility differences were not sufficient for them to reject the tumor, but they might have provided help to the

mutant p53-specific CTL in rejection of the tumor by the immunized mice. Therefore, we repeated the experiment in DBA/2 mice, and found only one of 3 immunized mice survived, compared to none of 3 control mice. This difference was certainly less impressive, suggesting that the minor histocompatibility differences might play a role. These experiments are being repeated with larger numbers of animals. In the meantime, however, we are also exploring other tumors that are of BALB/c origin that could be used as models.

We have also been able to generate CTL to another mutant p53 protein, T104, by immunizing with cells pulsed with the corresponding mutant peptide. These CTL kill tumor targets transfected with the T104 mutant p53 gene and not tumors expressing the T1272 mutant p53. Thus, these two mutations serve as good specificity controls for each other. However, the T104 synthetic peptide is hard to purify and work with, because it contains two cysteine residues that tend to cross-link molecules. This problem has been partially overcome by using the alpha-amino butyric acid analogue to replace cysteine as mentioned above, but preliminary evidence suggests that this substitution may affect the specificity of the response.

The other major cancer vaccine progress is in the human phase I/II clinical trial, which has now finally gotten under way. The IND was filed with the FDA at the end of November, 1993, and we received some suggestions for revisions on January 11, 1994. We carried out the requested stability and identity studies on the peptides, and the revised protocol was resubmitted to the IRB on February 11, 1994. After IRB and CTEP approval, it was forwarded to the FDA in early March, and finally approved by the FDA on March 23. We received the peptides for the first few identified patient tumor p53 and ras mutations in December, 1993, and early January, 1994. However, these are prepared as bulk powder and needed to be dissolved, filter sterilized, and vialed under GMP conditions by a contractor, who would also test the vialed peptide for sterility, pyrogenicity, and general safety (by injecting mice and monitoring their weight for two weeks). The contract was finally approved in January, and the vialing and testing begun. Since the vialing and testing of each peptide takes about 4 weeks, we received the first vialed peptides in March just in time to start the protocol when we received FDA approval. Our first patient was a 65-year old dentist from Dallas with metastatic cancer of the pancreas expressing a mutant ras protein, treated on March 31, 1994. Peripheral blood mononuclear cells were isolated from a blood sample from the patient, incubated with the specific mutant ras peptide for two hours, washed, irradiated, and reinfused into the patient intravenously. There were no adverse reactions noted clinically or on laboratory tests. He returned to NIH three weeks later for a second immunization. No adverse reactions were noted after the second immunization, and the patient had not developed an allergic hypersensitivity to the peptide caused by the first immunization. We have drawn blood to test the CTL response of the patient to the immunization, but do not have any results yet. It is too soon to expect a clinical response of the tumor yet either.

Five other peptides have now been synthesized and vialed. To date, a total of 26 tumors have had ras or p53 mutations characterized for this protocol, including breast, lung, colon, ovarian, and pancreatic cancers. These patients must be reexamined and also tested for immunologic anergy to be certain of eligibility for the treatment protocol. We have also been receiving hundreds of phone calls from interested patients or their physicians. Thus, there are a number of patients at various stages of evaluation that we hope to be able to enter into the trial. The trial is being carried out jointly with Dr. David Carbone and Dr. John Minna, U. Texas Southwestern in Dallas, and Drs. Bruce Johnson and Carmen Allegra of the NCI-Navy Medical Oncology Branch.

Honors and Awards:

McLaughlin Visiting Professorship, University of Texas, 1992
President, American Society for Clinical Investigation, 1993

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04024-07 MET

PERIOD COVERED
October 1, 1993 through September 30, 1994

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Gene Expression in Normal and Malignant Human Lymphocytes

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

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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
Vicki Seyfert, 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:	PROFESSIONAL:	OTHER:
6	5	1 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 genes which regulate the development and function of normal human B lymphocytes and on genes which deregulate this program and cause lymphoid malignancies. Subtractive hybridization cDNA libraries enriched for genes expressed specifically in human B cell lymphomas were used in conjunction with automated DNA sequencing to isolate novel human lymphoid-restricted genes. 1305 subtracted cDNAs were sequenced and one half of the sequences were from novel human genes. Approximately 9% of the sequences encoded novel proteins that showed impressive amino acid sequence similarity to previously cloned genes. Northern blot analysis was used to confirm the identification of over 30 novel human lymphoid-restricted genes.

Three of these novel lymphoid-restricted genes which encode nuclear factors were selected for intensive analysis. The first gene, LAF-4, is homologous to AF-4, a gene which is involved in the recurrent t(4,11) translocation found in pre-B cell acute lymphocytic leukemia (ALL). Functional studies of LAF-4 and AF-4 demonstrated that they define a novel transcription factor family. The second gene encodes a zinc finger transcription factor which has shown by several groups to be translocated in at least 20-30% of all cases of diffuse large cell lymphoma and has been termed BCL-6. The third gene, LySP100, is homologous to a nuclear autoantigen, SP100, which is localized to 5-15 discrete dots in the nucleus, termed "nuclear bodies". Although the function of nuclear bodies is unknown, it is intriguing that the product of the PML gene, which is involved in a characteristic t(15,17) translocation of acute promyelocytic leukemia, is also localized to this subnuclear organelle.

Project Description

Major Findings:

LAF-4

We initially identified the LAF-4 gene as a presumptive nuclear factor since our partial cDNA encoded a potential nuclear localization signal. Subsequently, we found LAF-4 to be homologous to AF-4, a gene which is fused to the MLL gene in the t(4;11) translocation of pre-B cell ALL. The MLL gene at 11q23 is homologous to the Drosophila regulatory protein trithorax and is translocated to more than ten different chromosomal loci in a variety of acute leukemias. Each translocation generates an in frame fusion protein between the amino terminus of MLL and the carboxy terminus of the fusion partner. Interestingly, the lineage of the acute leukemia that results from the translocation correlates well with the fusion partner: the t(4;11) MLL/AF-4 translocation is found almost exclusively in pre-B cell ALL whereas the t(9;11) MLL/AF-9 fusion is always found in acute myeloid leukemias. Since the region of MLL included in each fusion protein is virtually identical, the fusion partner appears to dictate the lineage restriction of the leukemia.

By cloning the full length 9 kb LAF-4 cDNA we found that AF-4 and LAF-4 are homologous over their entire coding regions yet neither protein is homologous to previously cloned genes. LAF-4 is highly conserved in evolution: the amino acid sequence of the mouse LAF-4 gene is greater than 95% identical to the human LAF-4 gene over the region analyzed thus far. Both the human and the mouse LAF-4 genes are expressed at highest levels in lymphocytes in the spleen and the thymus, at low levels in the brain and the lung and at undetectable levels elsewhere. In contrast, the expression pattern of AF-4 is more widespread.

Using rabbit antibodies to LAF-4, we found that LAF-4 was indeed a nuclear protein which displayed an unusual granular immunofluorescent staining pattern that is distinctly different from the diffuse nuclear staining pattern seen with sequence-specific DNA binding proteins. In fact, neither LAF-4 nor AF-4 contain any of the previously recognized DNA binding domains. Nevertheless, we have found that both LAF-4 and AF-4 have extraordinarily potent transcriptional activating domains. One of these transcriptional activating domains is retained in the MLL/AF-4 fusion protein and may contribute to the activity of the oncogene. Taken together, these results demonstrate that LAF-4 and AF-4 define a novel gene family which encodes nuclear proteins that are presumptive transcription factors.

BCL-6

Initially, we identified BCL-6 as a zinc finger gene in our subtracted library which was expressed at highest levels in human B cell tumor lines and at much lower levels in other cell types. Subsequently, several groups independently cloned BCL-6 and showed that it was translocated to a wide variety of other loci in at least 20-30% of cases of diffuse large cell lymphoma. These translocations do not alter the coding region of BCL-6 but invariably replace the 5' end of the gene contain its promoter with sequences from the translocation partner. The immunoglobulin loci are among the BCL-6 translocation partners and thus it seems likely that the expression of BCL-6 is disregulated due the juxtaposition with immunoglobuin promoters and enhancers. Since diffuse large cell lymphoma constitutes the largest sub-category of non-Hodgkins lymphomas, it is of considerable interest to study the mechanism of transformation of B lymphocytes by BCL-6 and the role of BCL-6 in normal B cell development.

BCL-6 is belongs to the Kruppel subfamily of zinc finger proteins and possesses six zinc fingers at its carboxy terminus. We have determined a binding site for BCL-6 by using its zinc finger DNA binding domain to select a site from a pool of random oligonucleotides. This approach yielded a

variety of closely related sequences that contain a 9 base pair core sequence that we have shown is sufficient for BCL-6 binding. In addition, BCL-6 has a 110 amino acid amino-terminal domain which is shared by a subset of zinc finger transcription factors as well as by other proteins which do not appear to be nuclear proteins. The function of this amino-terminal domain is unknown but its presence in a variety of developmentally important Drosophila transcription factors is intriguing. In several instances, the transcription factors that contain this amino-terminal domain have been shown to be transcriptional repressors making it interesting to determine the role of this amino-terminal domain in repression. We have isolated the mouse BCL-6 gene and find it to be extraordinarily well conserved at the amino acid level: no amino acid substitutions are present in the zinc finger domains and only one conservative change is present in the amino-terminal domain, again emphasizing the functional importance of these two domains.

We have found that the expression pattern of human and mouse BCL-6 mRNA is regulated in an intriguing manner. All tissues express a relatively low level of BCL-6, including the spleen and thymus. Some B cell lines, including most long-term lymphoblastoid cell lines transformed with Epstein-Barr virus, do not express any detectable BCL-6 mRNA. Nonetheless, most human B cell tumor lines express very high levels of BCL-6, including lines that have no detectable rearrangement of the gene. The high BCL-6 levels in B cell tumors may reflect a high level of BCL-6 at particular stages of normal B lymphocyte differentiation or activation. Alternatively, up-regulation of BCL-6 may be a frequent mechanism of oncogenic transformation of human B cells, even in lines that have not rearranged BCL-6.

LySP100

Our interest in the lymphoid-restricted gene LySP100 began with its strong homology to SP100, an nuclear autoantigen detected with sera from patients with primary biliary cirrhosis. SP100 has been localized to 5-15 discrete, sub-nuclear dots by immunofluorescence which correspond to a nuclear sub-structure that has be known to electron microscopists for decades as a "nuclear body". Nuclear bodies are ring-like structures approximately one-third the size of a nucleolus which consist of a proteinaceous, electron dense ring surrounding an central amorphous granular region. Nuclear bodies are found in all cell types, are highly conserved in evolution (they have been noted in insect cells) and are increased in size and number in many tumor cells and during activation of cells by a variety of agents including cytokines and hormones.

Interest in nuclear bodies has risen recently with the localization of the PML gene product to this nuclear substructure. The PML gene is fused to the retinoic acid receptor a (RARa) gene in the characteristic t(15;17) translocation of acute promyelocytic leukemia (APL). In APL cells, the normal nuclear bodies are disrupted by the presence of the PML/RARa fusion protein. Interestingly, treatment of APL cells with retinoic acid causes the reappearance of the nuclear bodies, the differentiation of the cells along the myeloid lineage and clinical remission of the tumor. Thus, it is conceivable that the disruption of nuclear bodies by the PML/RARa oncoprotein may contribute to the differentiation block and malignant transformation that occurs in APL cells.

The LySP100 gene is expressed predominantly in the B cell lineage whereas the expression of both SP100 and PML is widespread. Using confocal immunofluorescence microscopy, we demonstrated that LySP100 was also targeted to nuclear dots in lymphoid cells. In double immunofluorescence experiments, we made the unexpected observation that the PML-containing nuclear dots are entirely non-overlapping with the LySP100 dots. Furthermore, we confirmed the observations of other laboratories that PML and SP100 colocalize. Therefore, our analysis of LySP100 has revealed an unanticipated molecular heterogeneity in these nuclear substructures. Thus, although the function of nuclear bodies is currently unknown, our findings suggest that this function can be regulated in a cell type-specific fashion.

It has recently been shown that the transcriptional regulatory protein, ICP0, of herpes simplex virus (HSV) disrupts nuclear bodies. ICP0 does not appear to be a DNA binding protein but nevertheless transactivates a number of immediate early genes of HSV as well as several cellular genes. Furthermore, ICP0 is critical for the growth of HSV under conditions of low multiplicity of infection. These findings, taken together with the potential involvement of nuclear bodies in the pathogenesis of acute promyelocytic leukemia, implicate nuclear bodies as important regulatory sub-nuclear organelles and make the elucidation of their function very compelling.

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

PROJECT NUMBER
Z01 CB 08907-11 OD

PERIOD COVERED
October 1, 1993 to September 30, 1994

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. Wang	Visiting Associate	OD DCBDC NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Office of the Director

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	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.)

1. IL-4 regulation of gene expression and protein synthesis: The αCD3-induced cytotoxic response is regulated by protein kinase C (PKC). IL-4 circumvents the requirement of PKC and can either directly, or through the activation of an intermediate event(s), activate the perforin gene expression which leads to the production of cytolytic granules to generate killer cells. Thus it appears that cytokine can regulate cell function at the gene level.

2. Tumor-cell-induced suppression and reversal of suppression: An in vitro model was constructed to simulate the tumor-cell-induced suppression. Single suppressor agent-induced suppression could be reversed by an appropriate anti-suppressor agent. In contrast, multiple anti-suppressor agents were required to correct the suppression induced by multiple suppressor agents. In a syngeneic system, it was found that multiple anti-suppressor agents were required to correct tumor-cell-induced suppression, suggesting that tumor cells induce immune defects at multiple sites, and thus requires multiple approaches to restore the immunocompetence. This will have clinical implication in the immunotherapy of cancer.

3. Differential requirement of protein tyrosine kinase (PTK) and protein kinase C (PKC) in lymphocyte activation: The transduction pathway for the activation through IL-2 receptor was found to be primarily PTK-dependent. Activation through TCR-CD3 complex is a more complex event. Generation of proliferative response can employ either the PTK- or PKC-dependent pathway; nevertheless, generation of cytotoxic response is primarily PKC-dependent. A PTK-independent pathway does exist for the CD3-AK response, and PKC activation is not necessarily preceded by PTK activation. These findings will change our current thinking on the role of PTK and PKC in T cell activation.

Major Findings:

1. IL-4 regulation of perforin gene expression and BLT-esterase production in αCD3-induced activated killer cells: Generation of αCD3-induced activated killer cells CD3-AK in resting T cells is primarily PKC dependent and is blocked by the depletion or inhibition of PKC inhibitors. These changes are accompanied by the suppression of perforin gene expression (mRNA) and BLT-E production. However, adding IL-4 into the cultures restored the perforin mRNA expression and BLT-E production, and also the cytolytic activity of the CD3-AK cells. Furthermore, for preactivated CD3-AK cells cultured in IL-2, PKC inhibitors also suppressed the perforin mRNA and BLT-E with the concomitant reduction of cytolytic activity. Similar to the resting T cells, in the SSP-maintained preactivated CD3-AK cells, switching the cytokine from IL-2 to IL-4/IL-2 restored perforin mRNA expression and BLT-E production with concomitant restoration of the cytolytic activity. In contrast, switching from IL-4/IL-2 restored perforin mRNA expression and BLT-E production with concomitant restoration of the cytolytic activity. In contrast, switching from IL-4/IL-2 to IL-2 gave the opposite effect. These findings indicate that IL-4 may play a role in the late stage of αCD3 activation to regulate the expression of perforin gene and probably the translation process during the generation of anti-tumor activated killer cells.

2. Tumor-cell-induced suppression and reversal of suppression: A panel of suppressor agents which included CsA (cyclosporine A), SSP staurosporine), BSO (L-buthionine-[S,R]-sulfoximine) and PMA, and a panel of anti-suppressor agents which included IL-2, IL-4, GSH (glutathione) and amiloride, were tested. It has been determined that these suppressor/anti-suppressor agents acted differently on four specific sites of immune arm which affected the generation of T cell proliferative and cytotoxic responses to αCD3 activation. They included 1) IL-2 production, 2) PKC-regulated cytolytic granule production, 3) GSH-regulated maturation of functional granules, and 4) granule exocytosis. When a single suppressor agent was used, αCD3-induced proliferation was inhibited by CsA, BSO, and EL-4 tumor cells, whereas the generation of αCD3-inducted killer cells CD3-AK was inhibited by all suppressor agents tested. All suppressor agents except BSO and low dose PMA reduced the production of BLT-esterase, and PMA was found to reduce the granule exocytosis. Except for EL-4, suppression induced by a single suppressor agent could be corrected by an appropriate single anti-suppressor agent. Multiple suppressor agents induced profound suppression of CD3-AK response. In all cases except one, multiple anti-suppressor agents were required to correct the immune defects induced by multiple suppressor agents. Finally, EL-4 tumor-cell-induced immunosuppression could not be corrected by any single anti-suppressor agent tested, but a combination of IL-4, GSH and amiloride fully restored the CD3-AK response. These results suggest that tumor cells may induce multiple immune defects that require multiple anti-suppressor agents for correcting the defects to restore the host immunocompetence.

3. Differential requirement of protein tyrosine kinase (PTK) and protein kinase C (PKC) in the IL-2-induced LAK cells and αCD3-induced CD3-AK cell responses: Experiments were performed to examine the role of PTK and PKC in the signal transduction pathways for lymphocyte activation through IL-2R to generate LAK cells and through TCR-CD3 to generate CD3-AK cells. Two PTK

inhibitors (PTK-I, herbimycin A, and genistein) and two PKC inhibitors (PKC-I, calphositin C, and staurosporine) were used in the experiments. It was found that the primary activation pathway through IL-2R was PTK-dependent, that generation of both the IL-2-induced proliferative and cytotoxic responses were completely abrogated by PTK-I and not by PKC-I. Quite different results were obtained with the αCD3-induced CD3-AK cell response. First, the αCD3-induced proliferation was only partially inhibited by PTK-I or PKC-I alone. Secondly, generation of CD3-AK cytotoxic response was primarily PKC-dependent, that only PKC-I induced significant inhibition. Genistein was found to reduce protein tyrosine phosphorylation (PTP) both in the LAK cells and CD3-AK cells, indicating that CD3-AK cells were also susceptible to PTK-I treatment. Further studies showed that PTK-I and not PKC-I suppressed perforin mRNA expression and BLT-E production in LAK cells, and the opposite was true for CD3-AK cells. These results indicate that different pathways were employed in lymphocyte activation through IL-2R and TCR-CD3. The former is primarily PTK-dependent. Activation through TCR-CD3 is a more complex event. Induction of proliferative response can employ either PTK- or PKC-dependent pathway, whereas induction of cytotoxic response is primarily PKC dependent. Furthermore, it appears that a PTK-independent pathway exists for the induction of CD3-AK response, and thus implicates that activation of the second messenger PKC is not necessarily preceded by PTK activation.

4. Differential regulation by IL-4 of protein tyrosine phosphorylation (PTP) in IL-2-induced LAK cells and αCD3-induced CD3-AK cells and the correlation with their cytolytic activity: This study has examined the role of PTK in the IL-4 and/or IL-2 regulation of the generation of IL-2-induced cytolytic LAK cells and αCD3-induced cytolytic CD3-AK cells. IL-2 and αCD3 induced different patterns of PTP in LAK cells and CD3-AK cells. IL-4 down-regulated the PTP in LAK cells, but up-regulated the PTP in CD3-AK cells, in a dose dependent fashion. The effect of IL-4 on PTP did not appear to be selective for a particular kinase or substrate, and the changes covered a wide range of proteins from 175 to 35 kDa. The changes in PTP are correlated with the down- or up-regulation by IL-4 of the LAK cell or CD3-AK cell cytolytic activity, respectively, and the production of BLT-esterase. αIL-4 specifically blocked all the effects which were induced by IL-4. Adding PTK inhibitor genistein in cultures at 3 μg/ml, the maximal dose which could be tolerated by lymphocytes in cultures, completely inhibited the generation of cytolytic LAK cells but had no effect on the generation of CD3-AK cells which were cultured in IL-2 alone. However, genistein inhibited the IL-4 augmented cytolytic activity of CD3-AK cells. Again, these changes are closely correlated with the effect of genistein on PTP in LAK or CD3-AK cells. Genistein added at 3 μg/ml significantly reduced the PTP in LAK cells, and inhibited the IL-4 augmented PTP in CD3-AK cells to the basal level which was obtained with CD3-AK cultured in IL-2 alone. These findings indicate that PTK appears to be involved in the regulation of IL-2-induced LAK cell response and IL-4-mediated up- or down-regulation of CD3-AK or LAK cell response.

5. Function of PKC in the regulation of T cell activation: The screening of different PKC antagonists on the generation of αCD3-induced proliferative and

cytotoxic responses is nearly completed. Three groups of antagonists were identified in this tedious process. The first group of antagonists is highly specific for PKC, which includes calphostin C, and chelerythrine. The second group consists of antagonists which are selective for PKC but not highly specific, they include staurosporine and PMA. The third group of antagonists has anti-PKC activity, but may also have a variety of other pharmacological effects, such as amiloride. Other reagents which have been tested were either too toxic, or have much less selective activity on PKC; they include 9-aminoacridine, cardiotoxin, mastoparan, mellitin, polymyxin B, and sphingosine, etc. Thus, the five reagents, namely calphostin C, chelerythrine, staurosporine, PMA, and amiloride, are the drug of choice for studying the PKC biological functions.

Proposed Course of Study:

1. To continue the work on studying the biological function of PKC on the regulation of T cell activation.

2. To study the differential requirement and sequential involvement of PTK and PKC in lymphocyte activation.

3. To study cytokine regulation of T cell activation.

Publications:

Hargrove ME, Wang J, and Ting CC. Regulation by glutathione of the activation and differentiation of IL-4 dependent activated killer cells. Cell Immunol 1993;149:433-43.

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: NO1-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 sytem 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 working 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. 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 94 Funds: $75,195 D

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant baculoviruses and protein products using inserted tumor associated genes and/or cytokine genes.

Principal Investigator: Terry Mainprize Ph.D.
Performing Organization: Program Resources, Inc./DynCorp
City and State: Rockville, Maryland
Contract Number: N01-CB-21026-01
Starting Date: 9/30/92 **Expiration Date:** 12/30/93

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: PRI has been unable to produce purified CEA protein derived from the baculovirus-CEA construct on the scale required to meet contract requirements. Since two contract extension completion dates were not met, a mutually agreed upon ending settlement is being negotiated.

Significance to Cancer Research: This master agreement order would have provided clinical grade recombinant baculovirus derived CEA to be used in the potential treatment of cancer patients.

Project Officer: Kathleen Siler, M.S.
Program: Immunology Resource
Technical Review Group: Cancer Biology-Immunology Contracts Review Committee
FY94 Funds: 0 D

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant baculoviruses and protein products using inserted tumor associated genes and/or cytokine genes.

Principal Investigator: Terry Mainprize Ph.D.
Performing Organization: Program Resources, Inc./DynCorp
City and State: Rockville, Maryland
Contract Number: N01-CB-21026-02
Starting Date: 9/30/93 **Expiration Date:** 9/29/94

Goal: To produce clinical grade recombinant baculovirus derived Prostate Specific Antigen (PSA) protein that meet all FDA guidelines for patient administration.

Approach: PRI will produce PSA protein from a baculovirus-PSA 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 PSA protein as demonstrated by ELISA assay. The homogeneity of the PSA protein will be confirmed by SDS-PAGE gel analysis and confirmation of the reactive epitope will be demonstrated on Western blot analysis using an anti-PSA specific monoclonal antibody. 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: PRI has not been able to meet contract requirments for N01-CB-21026-01, therefore the recombinant baculovirus-PSA construct has not been given to PRI for vaccine production. PRI is currently negotiating with a third party subcontractor to perform the work for this Master Agreement Order.

Significance to Cancer Research: This master agreement order would provide clinical grade recombinant baculovirus derived PSA to be used in the potential treatment of cancer patients.

Project Officer: Kathleen Siler, M.S.
Program: Immunology Resource
Technical Review Group: Cancer Biology-Immunology Contracts Review Committee
FY94 Funds: 0 **D**

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant baculoviruses and protein products using inserted tumor associated genes and/or cytokine genes.

Principal Investigator: Terry Mainprize Ph.D.
Performing Organization: Program Resources, Inc./DynCorp
City and State: Rockville, Maryland
Contract Number: N01-CB-21026-03
Starting Date: 9/30/93 **Expiration Date:** 9/29/94

Goal: To produce clinical grade recombinant baculovirus derived ras protein containing the position 12 glycine→valine point mutation that meet all FDA guidelines for patient administration.

Approach: PRI will produce ras (gly→val) protein from a baculovirus-ras (gly→val) 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 ras (gly→val) protein as demonstrated by ELISA assay. The homogeneity of the ras (gly→val) protein will be confirmed by SDS-PAGE gel analysis and confirmation of the reactive epitope will be demonstrated on Western blot analysis using an anti-ras specific monoclonal antibody. 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: PRI has not been able to meet contract requirments for N01-CB-21026-01, therefore the recombinant baculovirus-ras (gly→val) construct has not been given to PRI for vaccine production. PRI is currently negotiating with a third party subcontractor to perform the work for this Master Agreement Order.

Significance to Cancer Research: This master agreement order would provide clinical grade recombinant baculovirus derived ras protein with the glycine→valine point mutation to be used in the potential treatment of cancer patients.

Project Officer: Kathleen Siler, M.S.
Program: Immunology Resource
Technical Review Group: Cancer Biology-Immunology Contracts Review Committee
FY94 Funds: 0 **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. Five transgenic mouse lines containing the *MMTVLTR-Int3*, *WAP-Int3*, *MMTVLTR-Wnt-1*, *MMTVLTR-TGFb*, and *MT-TGFa* transgenes are also being maintained. The *TGFa* "knockout" mouse strain has also recently been introduced into the colony. In addition, alimited breeding nucleus of the high-incidence C3H/OuJ, GR/imr, BALB/cfCZECHII, and BALB/cfMS inbred mouse strains and the low-incidence BALB/cp and FVB inbred mouse strains are maintained.

Progress: The transgenic and "knockout" mouse strains are being used to determine the consequences of transgene expression on mammary gland development and mammary tumorigenesis both individually and as F1 hybrids with the FVB3 (MMTV-Int3) transgenic mouse strain. In part the choice of transgenic (or knockout) mouse strains was based on our observation that mammary tumors arising in FVB3 mice overexpress TGFa. At the present time we are accumulating Int3/TGFa bitransgenic females and Int3/TGFa knockout females. Similarly, Wnt1 and FVB3 mice are being genetically crossed to determine whether thes two transgenes are sufficient for multifocal mammary tumorigenesis. We have discovered a new common insertion site for MMTV in a CZECHII outgrowth line (CZ21) 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 avtivation. 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 94 Funds: $120,195

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 prepares mice for the various studies carried out in the Laboratory of Genetics. 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, the silicone gel, and are working out the basic dosiometry 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. Using inbred SENCAR mice developed on the contract, we are studying the genetics of BALB/c (a skin tumor resistant strain) and SENCAR (a highly susceptible strain). Beginning with congenic strains carrying large segments of DBA/2 chromatin from chromosomes 1,4, and 11, we are developing recombinants that contain more restricted segments.

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 94 Funds: $993,669 B

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant vaccinia, other pox viruses, Salmonella, polio, adenovirus, and/or BCG with inserted tumor associated genes and/or cytokine genes.

Principal Investigator: Gail Mazzara, Ph.D.
Performing Organization: Therion Biologics Corporation
City and State: Cambridge, MA
Contract Number: N01-CB-21154-04
Starting Date: 9/30/93 **Expiration Date:** 12/14/94

Goal: To produce clinical grade recombinant vaccinia vaccine containing the gene for point mutated ras (glycine→valine, truncated, wobbled) at codon 12 that meet all FDA guidelines for patient administration.

Approach: Therion will construct a recombinant ras vaccinia virus containing the point mutated ras gene at codon 12 (glyine→valine, truncated, wobbled). 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 for the insertion of the truncated ras gene was constructed at the NCI. The vector contains the ras gene and lac Z gene allowing for the selection of recombinant virus using a colorimetric screening procedure. The plasmid vector was used to generate a clinical grade recombinant virus using the Wyeth vaccinia strain. Following plaque purification, all progeny plaques were blue, indicating homogeneity of the recombinant virus stock. Virus stocks were analyzed for genomic structure (by Southern blot analysis) and for expression of protein (by immunoblot analysis). Based on the results of these analyses, a single isolate was selected for further amplification. The recombinant virus, designated vT2044, was amplified to generate a small seed stock for expansion for use in the generation of the Manufacturer's Master Virus Stock (MVS) for vaccine production.

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
FY94 Funds: 0 **D**

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant vaccinia, other pox viruses, Salmonella, polio, adenovirus, and/or BCG with inserted tumor associated genes and/or cytokine genes.

Principal Investigator: Gail Mazzara, Ph.D.
Performing Organization: Therion Biologics Corporation
City and State: Cambridge, MA
Contract Number: N01-CB-21154-05
Starting Date: 10/1/93 **Expiration Date:** 12/1/94

Goal: To produce clinical grade recombinant vaccinia vaccine containing the genes for MUC-1 and B7 that meet all FDA guidelines for patient administration.

Approach: Therion will construct a recombinant vaccinia virus containing the genes for MUC-1 and human B7. 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 MUC-1/B7 vaccinia vaccine. The vaccine must contain the appropriate cDNA sequence for the inserted genes as demonstrated by Northern and/or Southern blot analysis. The recombinant vaccinia vaccine must contain specific reactive epitopes as demonstrated on Western blot analysis using a monoclonal antibodies specific for MUC-1 and B7. 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: Since neither the MUC-1 nor B7 gene had previously been expressed using recombinant vaccinia virus, Therion generated individual recombinants containing each of the genes to evaluate protein expression and genomic stability prior to generating the divalent recombinant construct. The human B7 gene was cloned into a plasmid vector and B7 expression was confirmed using Western blot analysis. The MUC-1 gene contains 60 base pair tandem repeats. In vaccinia virus, tandemly arranged7hn homologous sequences are genetically unstable; with the potential of obtaining a variety of novel truncated and expanded genes that contain varying numbers of repeat domains. Therion cloned the genes into a plasmid vector for insertion into vaccinia virus. Following plaque purification, the majority of viruses contained the full length MUC-1 gene, with 10 repeated domains, however, minor species containing genes with varying numbers of these domains were also present in each virus population. A plasmid vector for the simultaneous insertion of the MUC-1 and B7 genes, was generated. This plasmid was used to generate a clinical grade recombinant virus using the parental Wyeth vaccinia virus strain. The recombinant was purified using a colorimetric selection assay. Selected plaques were analyzed for genomic structure by Southern blot analysis and based on these results, a single isolate was selected for further amplification and production of a small seed stock for expansion for use in the generation of the Manufacturer's Master Virus Stock for vaccine production.

Significance to Cancer Research: This master agreement order will provide clinical grade recombinant MUC-1/B7 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
FY94 Funds: $199,916 **D**

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant vaccinia, other pox viruses, Salmonella, polio, adenovirus, and/or BCG with inserted tumor associated genes and/or cytokine genes.

Principal Investigator:	Enzo Paoletti, Ph.D.
Performing Organization:	Virogenetics Corporation
City and State:	Troy, NY
Contract Number:	N01-CB-21155-02
Starting Date: 10/1/93	**Expiration Date:** 3/15/95

Goal: To produce clinical grade recombinant avipox (ALVAC-based) vaccine containing the gene for human B7 that meet all FDA guidelines for patient administration.

Approach: Virogenetics will generate a recombinant avipox (ALVAC) construct containing the human B7 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-B7 vaccine must contain a specific reactive epitope as demonstrated on Western blot analysis using an anti-human B7 monoclonal antibody supplied by NCI. The ALVAC-B7 vaccine will be manufactured, vialed 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 the human B7 protein was supplied to Virogenetics by NCI. Virogenetics generated the donor plasmid containing the B7 coding sequence and this expression cassette was inserted into ALVAC by in vivo recombination between the plasmid donor and the ALVAC genomic DNA. A B7 gene-containing recombinant was identified by plaque hybridization with a DNA probe derived from the B7 coding sequence. The recombinant virus was plaque purified and amplified. Flow cytometric analysis with anti-human B7 monoclonal antibody confirmed B7 expression on the surface of ALVAC infected cells. Immunoprecipitation analysis with anti-human B7 monoclonal antibody also demonstrated B7 expression. The recombinant construct has been sent to Pasteur Merieux for generation of the Master Virus Stock (production batch) and for vaccine production.

Significance to Cancer Research: This master agreement order will provide clinical grade recombinant ALVAC-B7 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	
FY94 Funds:	$149,659	D

CONTRACT RESEARCH SUMMARY

Title: Production of clinical grade recombinant vaccinia, other pox viruses, Salmonella, polio, adenovirus, and/or BCG with inserted tumor associated genes and/or cytokine genes.

Principal Investigator: Enzo Paoletti, Ph.D.
Performing Organization: Virogenetics Corporation
City and State: Troy, NY
Contract Number: N01-CB-21155-03
Starting Date: 10/1/93 **Expiration Date:** 3/15/95

Goal: To produce clinical grade recombinant avipox (ALVAC-based) vaccine containing the carcinoembryonic antigen (CEA) gene and the gene for human B7 that meet all FDA guidelines for patient administration.

Approach: Virogenetics will generate a recombinant avipox (ALVAC) construct containing the CEA gene and the human B7 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 sequences for the inserted genes as demonstrated by Northern and/or Southern blot analysis. The recombinant ALVAC-CEA-B7 vaccine must contain specific reactive epitopes as demonstrated on Western blot analysis using an anti-human B7 monoclonal antibody and COL-1 (anti-CEA monoclonal antibody) supplied by NCI. The ALVAC-CEA-B7 vaccine will be manufactured, vialed 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 the human B7 protein was supplied to Virogenetics by NCI. Virogenetics generated the donor plasmid containing the B7 coding sequence and this expression cassette was inserted into ALVAC-CEA by in vivo recombination. A CEA/B7 gene containing recombinant was identified by plaque hybridization with DNA probes derived from the CEA and B7 coding sequences. The recombinant virus was plaque purified and amplified. Flow cytometric analysis with anti-human B7 monoclonal antibody and monoclonal antibody COL-1 confirmed both B7 and CEA surface expression. Western blot analysis with monoclonal antibody COL-1 demonstrated CEA expression. Immunoprecipitation analysis with anti-human B7 monoclonal antibody also demonstrated B7 expression. The recombinant construct has been sent to Pasteur Merieux for generation of the Master Virus Stock (production batch) and for vaccine production.

Significance to Cancer Research: This master agreement order will provide clinical grade recombinant ALVAC-CEA-B7 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
FY94 Funds: $149,659 **D**

CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Norman Beaudry
Performing Organization: Hazleton Biotechnologies Corp.
Vienna, Virginia

Contract Number: NO1-CB-7-1010, Starting Date: 6/30/87, Expiration Date: 6/29/93
NO1-CB-3-3036, Starting Date: 6/30/93, Expiration Date: 6/29/97

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 ELISA 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 ELISA assays. Elevated IL-2 receptor levels have been demonstrated in the sera of patients with HTLV-I-associated adult T-cell leukemia, HIV-associated AIDS, hairy cell B-cell leukemia or Hodgkin's disease. The assays for murine monoclonal antibodies, human anti-murine antibody responses, as well as human anti-humanized monoclonal antibodies have been developed and applied to the study of patients receiving IL-2 receptor directed therapy.

Significance to Cancer Research: These studies helped elucidate the abnormalities of the immune system associated with the development of cancer. They have assisted in the categorization of malignancies of the lymphoid system. They are required for therapeutic protocols involving the use of the anti-Tac and Mikβ1 monoclonal antibodies. 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 humanized antibodies to the IL-2Rα 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: Thomas A. Waldmann, M.D.
Program: Cancer Biology Resource
Technical Review Group: Ad Hoc Technical Review Group
FY: 1994 Funds: $291,093 B

CONTRACT RESEARCH SUMMARY

Title: Maintenance of an Animal Holding Facility and Provision of Associated Research Services

Principal Investigator:	Ms. Leanne DeNenno
Performing Organization:	Bioqual, Inc.
City and State:	Rockville, MD

Contract Number: NO1-CB-40533 (Successor to Contract NO1-CB-85607)
Starting Date: 11/01/93 Expiration Date: 10/31/97

Goal: Maintain colonies of up to about 7,500-10,000 inbred mice, 50 inbred rats, 50 hamsters, and 40 rabbits and carry out selected protocols with these animals as specified by the Project Officer. There animals are to be maintained in support of intramural research programs in the Experimental Immunology Branch, DCBDC, NCI.

Approach: Colonies of mice, rats, hamsters sand 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 5,000 mice, 100 rats, and 50 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 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: Ad Hoc Intramural Technical Review Group
FY 94 Funds: $744,329

CONTRACT RESEARCH SUMMARY

Title: Facility for Preparing and Housing Virus Infected Mice, Genetically Manipulated Mice and Chimeric Mice.

Principal Investigator:	Ms. Kinta Divan
Performing Orgainzation:	Bioqual, Inc.
City & State:	Rockville, MD

Contract Number: NO1-CB-40537 (Successor to Contract NO1-CB-85608)
Starting Date: 10-01-93 Expiration Date: 09-30-97

Goal: Performs a variety of in vitro experiments in mice (up to a colony of 3600 animals) that cannot be performed on the 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 perormed involving the transfer of normal and neoplastic cells, infection with virus, inocculations of combinations of cells and virus, irradiation with γ-rays, preparation 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 bone marrow transplantation, thymectomy, spleen cell transfers, immunizations, viral preparations, bleedings, grafting of thymuses and skin, tissue preparations for DNA analysis, and breeding, care and manipulation of SCID mice.

Significance to Cancer Reseaarch: 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 the use of infetious agents in NIH animal colonies. All of the protocols used in the facility are related to viral infection, genetic manipulation, and hemapoietic 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 94 FUNDS:	$528,561.

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