Intramural Activities

October 1, 1991- September 30, 1992

92 annual report

Division Of

Cancer Biology, Diagnosis, and Centers

NATIONAL CANCER INSTITUTE

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

ANNUAL REPORT

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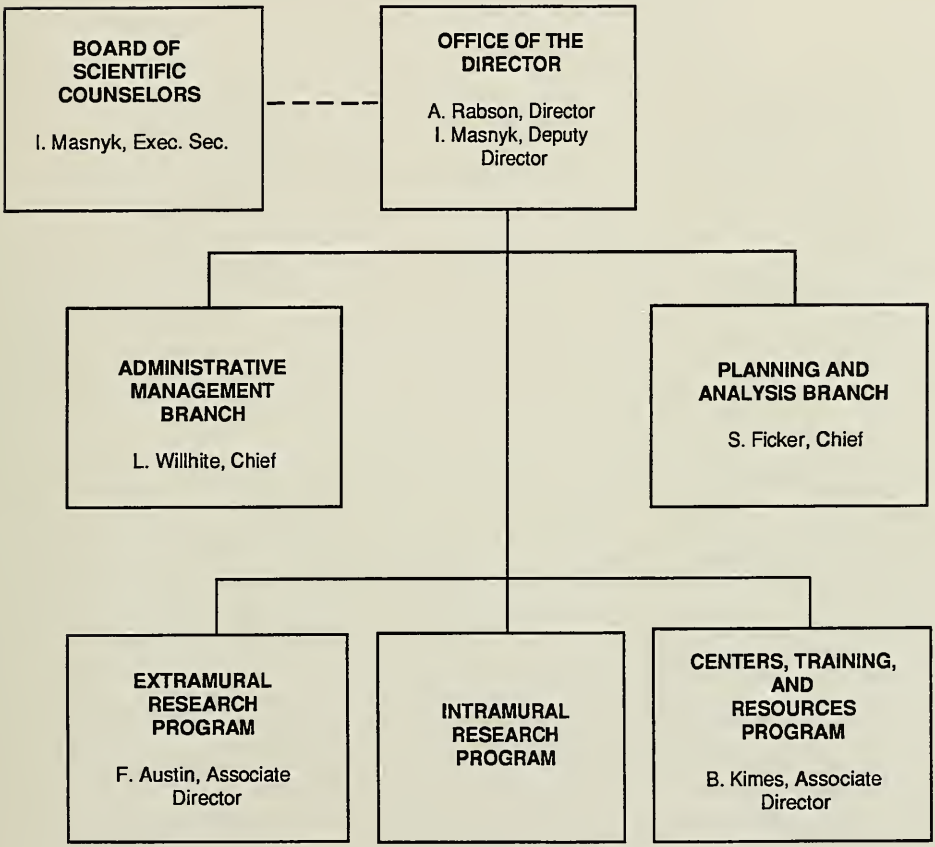

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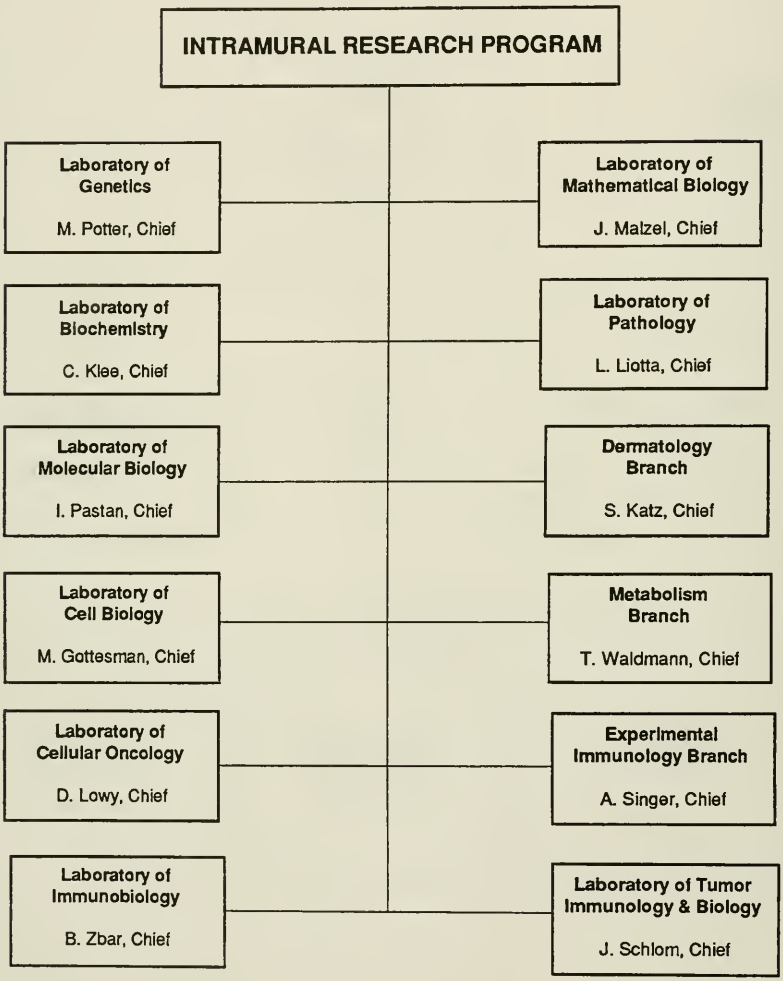

NATIONAL CANCER INSTITUTE
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1991 through September 30, 1992

INTRODUCTION

The Division of Cancer Biology, Diagnosis, and Centers supports laboratory and clinical investigations in cancer biology, immunology, and diagnosis. The Division is comprised of three research program areas. The Centers, Training and Resource Program consists of four branches: Cancer Centers, Construction, Organ Systems, and Research Training. The Extramural Research Program consists of three branches: Cancer Biology, Cancer Immunology, and Cancer Diagnosis. The Intramural Research Program consists of twelve laboratories and branches at the NIH Bethesda campus and at the Frederick Cancer Research and Development Center. They are the Laboratory of Genetics, the Laboratory of Biochemistry, the Laboratory of Molecular Biology, the Laboratory of Cell Biology, the Laboratory of Cellular Oncology, the Laboratory of Immunobiology, the Laboratory of Mathematical Biology, the Laboratory of Pathology, the Dermatology Branch, the Metabolism Branch, the Experimental Immunology Branch, and the Laboratory of Tumor Immunology and Biology. Each of these laboratories is directed by a scientist of international stature, 3 of whom are members of the National Academy of Science and 3 of whom are members of the Institute of Medicine.

Research conducted in the intramural laboratories covers a broad range of investigations from the regulation of gene expression in simple prokaryotic systems to clinical studies of human cancer. A common theme of many of these studies is to understand the molecular mechanisms involved in the regulation of normal cell growth and differentiation. The ultimate goal is to define the genetic changes responsible for neoplastic transformation, with special emphasis on understanding the genetic alterations associated with breast cancer. Recent advances in the identification of genetic markers associated with breast and other cancers are already providing useful in the diagnosis and prognosis of cancer. Considerable progress has been made during the past year in elucidating the molecular genetic mechanisms that result in tumor cell invasion, and in designing new agents that can interfere with the development of metastasis. One such agent, carboxyamido-imidazole (CAI), has been identified that blocks signal transduction and interferes with tumor cell motility. CAI shows particular promise for the treatment of breast and ovarian cancer. It is currently being evaluated in a Phase I trial. Studies of basic tumor cell biology and advances in molecular genetic technology have led to the development of another new class of cancer therapeutic agents in which cell recognition molecules (such as monoclonal antibodies and growth factors) are fused to genetically modified forms of a bacterial toxin, or are chelated to radionuclides to produce molecules capable of binding to tumor cells and killing them. Clinical studies are in progress to evaluate the efficacy of these approaches in the diagnosis and treatment of selected tumors. Advances in our understanding of the molecular mechanisms responsible for the development of the multidrug resistant phenotype, and the development of techniques of gene insertion have suggested entirely new

ways of overcoming the problems of life-threatening bone marrow toxicity associated with high dose chemotherapy.

A broad-based program in fundamental immunology explores the complex mechanisms governing the regulation of the immune response. The goal of these studies is to identify those aspects of the biology of tumor cells that can be enhanced to promote their recognition and attack by the immune system, as well as those elements of the immune system that can be stimulated to provide an effective antitumor response. Already, discoveries in fundamental immunology are suggesting entirely new ways to control or prevent the development of cancer. For example, CEA is a tumor-associated antigen expressed by most gastro-intestinal tumors, many adenocarcinomas of the lung, and over 50% of breast tumors. CEA alone is only weakly immunogenic; however, recent studies have shown that the co-presentation of CEA with a strong immunogen offers a way to induce anti-CEA responses for tumor immunotherapy. The gene for CEA has been introduced into a vaccinia virus, and the resulting construct is being evaluated for its ability to induce a specific immunotherapeutic response against CEA expressing tumors.

The diversity and high quality of all of these studies reflects the varied interests and training of the scientists and clinicians within the Division. Their continued interaction and exchange of ideas and technical expertise provides a fruitful environment for the development of exciting new strategies for the prevention, diagnosis, and treatment of cancer.

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

LABORATORY OF GENETICS

The focus of research activity in the Laboratory of Genetics, directed by Dr. Michael Potter, is to understand the genetic changes that occur in the neoplastic changes that occur in the development of plasma cell tumors. These include susceptibility and resistance genes that predispose to tumor formation, oncogenes, and genes that influence tumor development.

Pathogenesis of Plasma Cell Neoplasia: Resistance and Susceptibility Genes

Progress has been made in Dr. Potter's laboratory in the search for genes that determine susceptibility (S) and resistance (R) to the induction of plasmacytomas by chronic peritoneal irritants such as pristane. The model system on which much of the work is based is the S/R genes that determine resistance in DBA/2 inbred mice and susceptibility in BALB/cAn mice. Strong preliminary evidence that DBA/2 R genes were located on chromosome 4 has been confirmed. Mapping of the genes has been made possible by Dr. Beverly Mock, who has identified four new allelomorphic genes and screened and assembled potential genes described in other laboratories. The current evidence indicates there are two DBA/2 plasmacytoma (Pct)-R genes on the distal end of chromosome 4. Dr. Mock also has developed an assay for the susceptibility phenotype and shown that the S genes are also on chromosome 4. Much of this analysis involves the use of a panel of BALB/cAn.DBA chromosome-4 congenic strains that have been developed in the laboratory. Several of these strains have a strong partial resistance phenotype. Work continues on the identification of the physiological properties of the Pct-S/R genes. The hypothesis is that some of these genes increase the probability that B-cells in BALB/cAn mice are more prone to developing chromosomal rearrangements affecting the c-myc gene. Preliminary evidence suggests that the C.D2-chromosome-4 congenics have partial resistance to the DBA/2 efficient DNA repair phenotype.

Lymphocyte Circulation - Plasmacytoma Genesis

Dr. Stuart Rudikoff's laboratory examined the cellular basis for resistance to plasmacytoma induction by transferring BALB/c (S) or DBA/2 (R) bone marrow to severe combined immunodeficient (SCID) mice and injecting the chimeras with either pristane or the retrovirus J3V1 containing deregulated myc and raf oncogenes. Their studies have shown that DBA resistance cannot be overcome by the susceptible SCID microenvironment, suggesting that R/S in this system is determined by the donor bone marrow. They are also exploring the mechanism of immunological reconstitution of SCID mice using selective populations of cells. They have found differences in potentialities of peripheral lymph node and Peyer's patch lymphocytes to reconstitute the immune systems of these animals.

Organization and Control of Genetic Material in Plasmacytomas

Deregulated expression of myc secondary to chromosome translocation has been shown to be one essential component of the genetic alterations involved in pristene-induced plasmacytomas in BALB/c mice. Some of these myc-activating translocations occur 200-300 kb 3' of c-myc in a region called Pvt-1. Dr. Frederic Mushinski and his colleagues have shown that this locus is transcribed at very low levels in normal cells, but in much higher amounts in some plasmacytomas and in certain B lymphocytic cell lines in which both c-myc and Pvt-1 genes are amplified. Normal splenic lymphocytes and NIH3T3 cells increase their expression of Pvt-1 following mitogenic stimulation, suggesting that Pvt-1

is a new member of the "early response" genes, although its function remains unknown. Human and mouse homologies have been detected, indicating evolutionary conservation and implying a function that is essential to normal growth or development.

Drs. Linda Wolff and Katherine Nason-Burchenal, using a probe to the retroviral-myb insertion site that activates c-myb in murine promonocytic leukemias, have detected by PCR methodology the presence of the insertions as soon as 3 weeks after viral infection. These "mutant" cells represent a preneoplastic change in myeloid cells, and this work opens the possibility for studying in detail the pathogenesis of promonocytic leukemia in mice.

LABORATORY OF BIOCHEMISTRY

Investigators in the Laboratory of Biochemistry, directed by Dr. Claude Klee, conduct diverse research projects which span a broad array of disciplines encompassing molecular and developmental biology, genetics, and biochemistry. A new Drosophila genetics facility was created during the past year to support the work of several groups who use genetic techniques in Drosophila to study the function of proteins in vivo.

Regulation of Gene Expression

Regulation of gene expression remains a major focus of interest. Dr. Mark Mortin, who recently joined the Laboratory, has focused his research on studies of the regulation of RNA polymerase II, which is required for the synthesis of all messenger RNAs and is a prerequisite for regulation of gene expression. Both the structure of this multimeric enzyme and its mechanism of action are poorly understood. Dr. Mortin is using a genetic approach to identify proteins (and the protein subunits) that interact with Drosophila RNA polymerase II.

Dr. Carl Wu and his colleagues have continued to make original contributions to the study of three sequence-specific DNA-binding proteins in Drosophila, the heat shock transcription factor, HSF, and two factors which regulate the segmentation gene fushi tarazu, FTZ-F1 and tramtrack. Following the cloning of the HSF gene from humans in the previous year, they undertook a comparative study of HSF function in humans and Drosophila. Their studies have demonstrated 1) the importance of the C-terminal leucine zipper motif in maintaining the inactive HSF conformation, 2) the tramtrack protein functions as a repressor of the ftz gene, and 3) a new function for FTZ-F1 as an intermediate regulator of genes expressed at the onset of metamorphosis. These findings suggest that many of the transcription factors regulating developmental genes are utilized repeatedly throughout the life of the organism to serve different regulatory pathways.

The regulation and mode of action of the MyoD family of myogenic regulatory factors in the vertebrates and in Drosophila continues to be the focus of interest of Dr. Bruce Paterson's group. They have isolated four of the avian myogenic factor genes which are homologous to mouse MyoD, myogenin, myf5, and MRF4. During his sabbatical leave in Dr. Walter Gehring's laboratory in Basel, Switzerland, Dr. Paterson isolated and characterized the single Drosophila homologue, Dmyd, in order to take a genetic approach to study myogenesis.

Research in Dr. Charles Vinson's laboratory centers on the study of protein-DNA interactions. His present work focuses on two DNA binding motifs: the bZIP and the bHLH-Zip motif, motifs for which he has proposed structural models that he is

presently testing. Another goal of his group is to identify the specificity rules for leucine zipper dimerization. These rules will allow Dr. Vinson to design dominant-negative molecules that can be introduced into a biological context and disrupt the function of endogenous leucine zipper-containing molecules.

Dr. Dean Hamer's group has completed a ten-year study of gene regulation by metal ions with the discovery of a new nuclear regulatory factor, UPC31, that activates the reduction and uptake of Cu ions by yeast cells. Interestingly, the amino-terminal domain of UPC31 is homologous to the Cu- and DNA-binding domain of ACE1, the Cu-dependent activator of metallothionein (CUP1) gene transcription in yeast. Thus the basic pathway for Cu homeostasis in yeast cells is now known: Cu → UPC31 → uptake & utilization → ACE1 → CUP1 → sequestration. A remarkable feature of this pathway is that the three critical proteins - UPC31, ACE1 and CUP1 - all share a common structural feature, the "copper-fist."

The role of DNA methylation in the modulation of gene expression is a major focus of interest in Dr. Edward Kuff's laboratory. As an experimental system, his group is using a type of envelope-deficient mouse retrovirus, the intracisternal A-particle (IAP). The promoter activity of IAP long terminal repeats (LTRs) is inhibited both in vivo and in cell-free systems by methylation of specific CpG sites located in sequence motifs that are binding sites for nuclear factors. Previously this group showed that one of these factors, a heterodimeric protein designated EBP-80, is indistinguishable from a protein, Ku, that binds tightly to the ends of duplex DNA. Dr. Kuff's group has now shown that EBP-80/Ku can also recognize single to double strand transitions in DNA.

Proteins and the Control of Cellular Processes

Different approaches to the regulation of cell function are taken by other groups in the Laboratory who study specific proteins involved in different cellular processes. Dr. Shelby Berger and her colleagues are making steady progress in understanding prothymosin α gene expression and its essential role in cell division. The protein is encoded in a gene family which consists of one functional gene and five processed pseudogenes. The functional gene is alternatively spliced to yield two transcripts, the most common of which violates consensus rules for selection of the splice acceptor site. As a result of their studies, the Berger laboratory has refined the rules for splice acceptor selection. They believe that a G residue immediately upstream of an AG dinucleotide abrogates splicing except when two GAG motifs occur in tandem. The site at which the protein(s) are post-translationally modified has been identified. Current efforts are underway to identify the putative role of c-myc in the selective transcriptional activation of prothymosin α.

The long-term commitment of Dr. Claude Klee's group to the study of the activation of the calmodulin-regulated protein phosphatase, calcineurin, by calmodulin was rewarded last year by the report of Dr. Schreiber and his colleagues at Harvard that the inhibition of calcineurin's phosphatase activity by immunosuppressants is responsible for their inhibition of T cell activation. Collaborative studies have shown that immunosuppressants inhibit calcineurin in vivo and that inhibition of calcineurin activity parallels their inhibition of IL-2 expression. The immunosuppressant binding site has been localized to the carboxyl terminal two-thirds of calcineurin A which also harbors the catalytic center and the calcineurin B-binding site. Another important development has been the elucidation of the structure of the complex of calmodulin with a

calmodulin-binding peptide. These studies helped to define structural requirements for calmodulin target protein interactions and revealed the importance of the flexibility of the central helix in this interaction.

Organization of the Human Genome

Drs. Wesley McBride and Maxine Singer continue to devote their efforts to the study of the organization of the human genome. Dr. McBride's research in human genetics is focused in three areas: 1) the chromosomal localization of cloned genes, tumor breakpoints, and other cloned sequences; twenty additional cloned genes have been mapped during the past year; 2) the use of linkage analysis for mapping and identifying specific tumor susceptibility and other disease genes in families segregating these genes; collaborative projects include the mapping of the neurofibromatosis 2 (NF2) gene, an ovarian tumor translocation gene on 11q, an antigen recognized by cytolytic lymphocytes in human melanoma, and the mapping of disease genes in several genetic skin diseases; and 3) the construction of high resolution genetic and physical chromosome maps. Six dispersed highly polymorphic multisatellite markers have been identified in chromosome 22 which were subsequently used for linkage analysis in the Center for the Study of Human Polymorphism (CEPH) and NF2 families.

Dr. Singer and her colleagues are characterizing p40, the protein encoded in ORF1 of the L1Hs (human) retrotransposon. The polypeptide is cytoplasmic, phosphorylated, and includes a leucine zipper region capable, in principle, of forming a coiled-coil dimer. Complexes of p40 have been detected which could include homo- or heteromultimers. Four protein-binding sites have been identified in the 5'UTR.

DNA Replication

Studies of plasmid maintenance in the laboratories of Drs. Michael Yarmolinsky and Dhruba Chattoraj have focused on the molecular events involved in replication and partition of the archetypical low-copy-number plasmid, P1. These studies have established that the function of the bacterial heat shock proteins, DnaJ, DnaK and GrpE, is promoting P1 plasmid replication is limited to an activation of the plasmid-encoded initiator, RepA, for binding to the P1 origin. This activation involves the refolding of monomeric RepA protein. There is a cooperative interaction of RepA with the bacterial initiator, DnaA, and it is the latter protein that appears responsible for a strand opening which triggers initiation of replication. Studies of partition have challenged established concepts about the structure and function of the P1 partition module and provided new tools to enable the construction of a coherent model of partition.

LABORATORY OF MOLECULAR BIOLOGY

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

Immunotoxin and Oncotoxin Therapy of Cancer

To develop new cytotoxic agents for cancer treatment, Drs. Ira Pastan, David FitzGerald, and their colleagues have attached Pseudomonas exotoxin (PE) and genetically modified forms of PE to monoclonal antibodies (Mabs) or growth

factors to create cell specific cytotoxic agents. A mutant form of PE, LysPE38, has been chemically attached to MAbB3, an antibody that reacts with multiple mucinous carcinomas, including colon, some breast and some ovarian cancers. Indium labeled B3 has been prepared and shown to be a very effective imaging agent using human tumors growing in nude mice. Single chain recombinant immunotoxins have been made with antibodies that react with the erb-B2 oncogene (which is expressed in many human breast cancers), with an antibody (C242) that binds with many human colon cancers, and with antiTac that binds to the IL2 receptor present in many lymphomas and leukemias. These agents are in preclinical development. Other immunotoxins have been prepared that react with B cell lymphomas and the p75 subunit of the IL2 receptor. Recombinant toxins with longer half lives have been created by inserting the C_H2 domain of human IgG1 between the ligand and the toxin domains.

Intensive studies are currently in progress to determine more precisely the mechanism of action of PE cell killing; in particular, how PE is processed to an active C-terminal fragment which translocates to the cell cytoplasm and inhibits protein synthesis by ADP-ribosylating EF-2. These studies should suggest ways of modifying the toxic molecule to develop improved immunotoxins. In another series of investigations Dr. Kai Chang has isolated MAbK1 which reacts with many nonmucinous ovarian cancers, mesotheliomas and some squamous cell carcinomas. cDNAs encoding the K1 antigen have been isolated and are currently being sequenced. The K1 antigen is poorly internalized so that K1-PE40 is not very cytotoxic to target cells. The cDNAs encoding the light and heavy chains of K1 have been isolated and a single chain immunotoxin constructed, in an attempt to develop a more cytotoxic immunotoxin. In addition, Fab fragments have been made in E. coli and are being developed for use in imaging studies.

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells

The role of drug resistance in human cancer has been studied in collaboration with Dr. Michael Gottesman. Resistance to chemotherapy of human cancers has been studied by establishing in vitro and in vivo genetic systems which mimic development of drug resistance. Mechanistic studies on P-glycoprotein suggest that it is involved in removing drug directly from the plasma membrane. To study mechanisms, many mutants have been generated, and biochemical studies have been initiated including the purification of P-glycoprotein and reconstruction of drug-dependent ATPase activity in artificial liposomes.

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

Dr. Glenn Merlino and colleagues use transgenic technology, in which foreign DNA is stably introduced into the mammalian germ line, to investigate the role of growth factors, receptors, and oncogenes in the initiation and development of neoplasia, and to establish useful animal models to study the pathogenesis of human disease. Transgenic mice were made bearing the human TGFα gene. TGFα overexpression was found to induce a variety of abnormalities including mammary adenocarcinoma and hepatocellular carcinoma. Detailed molecular analysis of transgenic liver neoplasia has shown that TGFα promotes tumor formation and plays a role in tumor progression. Transgenic mice made using an activated form of an EGF-related gene, int-3, develop severe hyperplastic lesions of secretory glands and neoplasia of the salivary and mammary glands. They were arrested in development; the females were lactation deficient, and the male transgenic mice

were sterile due to epididymal hyperplasia. These findings demonstrate that expression of the activated int-3 gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia.

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

Dr. Sankar Adhya and colleagues have shown that each of the two promoters of the gal operon of E. coli is negatively regulated by the classical Gal repressor and Gal isorepressor. Both act by binding to the same two spatially separated operators, O_E and O_1. Gal repressor and isorepressor belong to a family of bacterial regulatory proteins, termed GalR family. An alignment of the proteins of the family show 60% homology throughout the entire sequences. It has recently been established that both repressor and isorepressor participate in the modulation of a gal region, which includes the mgl operon encoding one of the galactose transport systems and galR and galS, the genes for Gal repressor and isorepressor, respectively. The isorepressor has a major and the repressor a minor role on the negative control of the mgl operon. The latter carries a gal operator element at the -60 region. The effect of isorepressor on mgl is a the level of transcription initiation.

Dr. Adhya's group has also developed an in vitro transcription assay to study regulation with purified proteins. In this system, using DNA minicircles containing the gal promoters with lac operator sequences at O_E and O_1, they have demonstrated the requirement of DNA looping in vitro. Repression mediated by DNA looping inhibited the synthesis of complete as well as abortive transcripts, demonstrating that the repression was on the formation or activity of the initial transcribing complex.

Transcription Mechanisms and Structure-Function Analysis of RNA

Polymerase of E. coli

The overall goal of Dr. Din Jin's laboratory is to dissect the catalytic center and the regulatory domain(s) of RNA polymerase of Escherichia coli to study the mechanisms of transcription. The effect of mutations in RNA polymerase that confer rifampicin resistance (rif^r) on different steps of transcription have been analyzed. RNA polymerases with altered rifampicin binding are likely to be also altered in some vital transcription processes. By studying the correlation between a particular rif^r RNA polymerase and its altered transcription property, it should be possible to assign the functional role of the rifampicin binding site(s) of RNA polymerase.

Bacterial Functions Involved in Cell Growth Control

Dr. Susan Gottesman and colleagues have been studying the role of protein degradation in regulating gene expression and have continued with studies on the linkages between chromosome synthesis and partition of chromosomes during cell division. As part of their studies of the regulation of capsular polysaccharide synthesis by the unstable regulator, rcsA, they have found that a downstream trans-acting regulator of rcsA synthesis encodes RNAs which apparently mediate the regulatory effect. Mutations in both the regulator and target will allow further characterization of the mode of regulation. Mutations in the putative phosphorylation site of RcsB support a role for phosphorylation in capsule regulation. Dr. Gottesman's group is investigating the role of the heat shock

chaperone proteins in degradation of substrates of the Lon ATP-dependent protease and initiating studies on the recognition specificity of Lon. Studies on the role and regulation of the Clp energy-dependent protease have concentrated on developing strains for the tight regulation of Clp synthesis, and investigating the function of a newly discovered alternate regulatory subunit, ClpX.

Molecular Modeling

The main research interest of Dr. B.K. Lee, who recently joined the Laboratory of Molecular Biology, is to study by various theoretical means the forces that govern the structure and interaction of globular protein molecules, to predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties.

LABORATORY OF CELL BIOLOGY

The Laboratory of Cell Biology, directed by Dr. Michael Gottesman, is composed of the Molecular Cell Genetics Section, which focuses on studies of multidrug resistance, and the Chemistry Section, which focuses on studies of antigen processing.

Resistance of Cancer Cells to Anti-Cancer Drugs

Dr. Gottesman's laboratory, in collaboration with Dr. Ira Pastan, has emphasized studies on resistance to natural product anticancer drugs and cis-platinum. The major mechanism of resistance to natural product drugs is expression of the MDR1 gene which encodes the 170,000 dalton P-glycoprotein, an energy-dependent multidrug efflux pump. Analysis of this multidrug transporter through photoaffinity labelling studies, mutational alterations, and kinetic studies of drug transport has led to a model in which natural product hydrophobic drugs are removed directly from the plasma membrane. Two general strategies are being explored to exploit our knowledge of the multidrug transporter to improve chemotherapy. In the first approach, agents which inhibit the activity of the multidrug transporter have been studied in an MDR1 transgenic mouse system. In the second approach, retroviral vectors encoding the MDR1 gene are being used to confer a multidrug-resistant phenotype on chemotherapy-sensitive tissues such as bone marrow to allow dose intensification of anti-cancer drugs. Clinical testing of this approach in cancer patients is expected to begin in 1993.

The Mechanism of Antigen Processing

Research by Dr. Ettore Appella has utilized the technique of microcapillary high-performance liquid chromatography/tandem mass spectrometry to fractionate and sequence subpicolar quantities of natural peptides bound to class I and class II major histocompatibility complex (MHC) molecules. The sequence of eight different peptides bound to human class I MHC HLA-A2.1 and nine peptides associated with the MHC class II I-A^d has been determined. The structure of a cellular transcription factor, important as a control element of transcription of the HIV-1 virus, has been determined and experiments are being carried out with a nucleocapsid protein of 71 amino acids of HIV-1 to assess its binding properties to RNA and unravel the various stages of viral replication.

LABORATORY OF CELLULAR ONCOLOGY

The Laboratory of Cellular Oncology, directed by Dr. Douglas Lowy, carries out fundamental research on the cellular and molecular basis of neoplasia. Two major areas are being studied; ras-encoded proteins, and papillomaviruses.

Tumor Gene Expression In Vitro and In Vivo

Chimeras between ras and rap1A, which encodes a ras-like protein that can suppress ras-transformed cells, have been analyzed. Some chimeras were sensitive to ras-GAP, but resistant to NF1, and others were sensitive to cytoplasmic rap-GAP, but resistant to membrane rap-GAP. In NIH 3T3 cells, chimeras carrying the p21ras effector region and sensitive only to ras-GAP or only to cytoplasmic rap-GAP were poorly transforming. Thus distinct amino acids of p21ras and p21rap1A mediate sensitivity to each of the proteins with GAP activity, and ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap1A, respectively, in NIH 3T3 cells.

This group also identified and characterized the NF1 protein in mammalian cells. In cell lines derived from malignant schwannomas that arose in patients with NF1 disease (von Recklinghausen's neurofibromatosis), the levels of NF1 protein were found to be very low and the proportion of ras in the active GTP-bound form was high, although there were no mutations in the ras protein. The results are consistent with NF1 being a tumor suppressor gene that negatively regulates ras and show that ras proteins can be activated by defective regulation, as well as by mutational activation.

Analysis of Papillomaviruses

There is a strong association between malignant progression of human genital lesions and certain types of human papillomavirus types (HPV), most frequently HPV16. Previous work by Drs. Lowy and John Schiller established that PVs encode three transforming genes: E5, E6 and E7. Studies from other laboratories have shown that E6 binds the tumor suppressor protein p53 and induces its degradation in vitro. Drs. Lowy and Schiller have now determined that E6 decreases the in vivo half-life of p53 in human keratinocytes that express HPV16 E6. Further studies have shown that E6 has both p53 dependent and independent activities.

In other studies, this group has shown that BPV E5 induces the ligand independent activation of growth factor receptors. They have now constructed a series of PDGF and EGF receptor chimeras to examine the receptor domains responsible for this activation. Their results suggest that E5 activates the two receptors by different mechanisms. Dr. Lowy and his colleagues have begun to identify and characterize a cellular protein that specifically binds E5. Biochemical analysis and partial protein sequencing indicate that it is a new member of a family of proteins involved in intracellular protein trafficking.

Analysis of the structural and immunogenic features of PVs has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, the L1 major capsid proteins of BPV1 and HPV16 have been expressed via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into PV virion-like structures. The self assembled BPV L1 resembled intact virions and were able to induce high titer neutralizing antiserum. These results indicate that L1 has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious

virions. This is highly significant because this type of L1 preparation may be useful in a vaccine to prevent PV infection.

LABORATORY OF IMMUNOBIOLOGY

Research activities in the Laboratory of Immunobiology, directed by Dr. Berton Zbar, are focused in two major areas: the genetic basis of human renal cell carcinoma, and in chemotactic factors involved in inflammation.

The Genetic Basis of Human Renal Cell Carcinoma

Von Hippel-Lindau disease (VHL) is an autosomal dominant, multisystem neoplastic disorder. Dr. Zbar's group has located the VHL gene in a 6-8 Cm interval between RAF1 and D3S18. During the past year they have prepared a long-range restriction map around VHL. The map covers 2.4 megabases. They examined 75 unrelated individuals for evidence of a rearrangement in the VHL gene region. One family was identified in which renal cell carcinoma is inherited as an autosomal dominant trait. The gene which produces renal cell carcinoma in this family appears to be distinct from the VHL gene. Ten individuals in this family have been shown to have renal cell carcinoma. The translocation breakpoint in the t3;8 RCC family was mapped between D3S1187 and PTPγ loci in an -2cM interval.

Under a grant from the National Center for Human Genome Research, Dr. Zbar's laboratory has assisted in the preparation of a high resolution genetic map of human chromosome 3. To date, 122 loci have been placed in the short arm of chromosome 3, and 40 loci have been placed on the long arm of chromosome 3.

The Role of Chemotactic Factors in the Immune Response

Dr. Edward Leonard's group has continued to study the biochemistry of chemotactic factors secreted in response to inflammatory stimuli, and to characterize a serum protein that modulates macrophage motility. Macrophage stimulating protein (MSP) belongs to the group of *kringle* proteins that includes hepatocyte growth factor and proteolytic enzymes of the coagulation system. By in situ hybridization, the MSP gene has been mapped to chromosome 1. Hybridization of human MSP cDNA to animal genomic DNAs suggests that MSP is highly conserved among species.

Studies to further characterize and to determine the biologic significance of neutrophil attractant protein-1 (NAP-1 or IL-8) and monocyte chemoattractant protein-1 (MCP-1) are in progress.

LABORATORY OF MATHEMATICAL BIOLOGY

Research in the Laboratory of Mathematical Biology (LMMB), directed by Dr. Jacob Maizel, covers a broad range of theoretical and experimental studies of biological systems. Basic understanding of these biological systems serves as models for aspects of malignant and other disease processes. Many of the theoretical studies are only possible by using the supercomputing facilities at the Biomedical Supercomputing Center, Frederick Cancer Research and Development Center.

Sequence Analyses in Molecular, Viral and Cell Biology

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 and trends.

Supercomputing facilities were successfully upgraded to include a Cray YMP with 8 processors and 128 MW of real memory, an "on-line" mass storage tape facility, Convex minisupercomputer to manage mass storage, massively parallel sequence matching computer (MasPar 8000 processors), Vax 6620 front-end computers, and other peripherals. This is the only such supercomputing facility dedicated to biomedical research.

Dr. Maizel and his colleagues have developed new analytical tools for studies of proteins and nucleic acids. RNA structures up to 9433 bases in size have been predicted. RNA secondary structure methods have been refined to include alternate energy parameters, extended Monte Carlo simulations, and comparative studies to establish firmly the uncommon structural features in subregions of a number of sequences of HIV and other retroviruses. These predicted structural features have been correlated with biological features, leading to deeper understanding of the replication and expression processes in this group of viruses. In HIV-1 and related viruses, predicted stable features have been correlated with sites of tat-regulation elements, rev responsive elements and sites of translational frame-shift. Conserved secondary structure is predicted to be absent in regions of hypervariability in the envelope gene m-RNA's. Use of the supercomputer has allowed development of a look-up procedure for predicting stability of random sequences, which accelerates surveys nearly 100-fold. Monte Carlo techniques are being developed that yield greater than 80% correct prediction of t-RNA structures for more than 100 examined sequences. Theoretical folding of the entire genome of HIV-1 on the Cray YMP 8/128 gives a global structure that retains all of the previously noted local structures.

Information Theory in Molecular Biology

Information theory is being used by Dr. Thomas Schneider's group to understand molecular sequence patterns in genetic control systems. The first results showed that most binding sites contain just as much information as is required for them to be located in the genome. A graphical technique, called 'sequence logos,' was invented which helps to visualize the patterns at binding sites and is superior to the consensus sequences. The sequenced logos are now being used to study the fine structure of binding sites. The fundamental processes of transcriptional control, translation, DNA replication and partition of DNA to daughter cells are actively being studied by using these techniques.

Molecular Structure

Another focus of research activity is the study of the properties of biological macromolecules, including the physical chemistry of processes such as folding, binding and conformational changes. The ultimate goal is to develop methods to facilitate the study of larger molecular assemblies.

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. Using this approach, Dr. Robert Jernigan and his colleagues have been able to enumerate all of the possible folded topologies for several small proteins and to evaluate them with simple residue-residue interactions. A similar approach has proven useful for studying tertiary folding of RNA, and for investigating the binding of small peptides to larger proteins. These studies may lead to new methods of drug design.

Molecular Modeling

Molecular modeling has continued in four areas: membrane proteins, small peptides, DNA helices, and DNA-protein interactions. Conformational models have been developed for the antibiotic magainin and cecropins; these models have improved our understanding of how these molecules lyse cells and form channels.

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. One unresolved problem is the role of conformation in gene regulation; for DNA-protein interactions, these flexibilities and their asymmetries appear to play important roles. Other DNA forms, such as three-stranded helices and alternative base pairs, are also being modelled and their function in recombination is being investigated.

Image Processing

The genetic algorithm and the MasPar architecture have been integrated into the heterogeneous system that is being developed for RNA structure analysis. The system includes the facilities for activating algorithms that may run on various computer architectures that are accessible over a computer network. The RNA structure analysis system has also expanded to include more functionality for analyzing RNA conformations from various perspectives. This has involved the further enhancement of algorithms to explore secondary and tertiary structural motifs.

The termination structures of lambda TR2 have been extensively studied and the 5' noncoding regions of Polio-virus and its mutational relationships to RNA secondary and tertiary structures are being characterized in regard to its functionality. The system has been used to study the fine structural details of the HIV-1 rev responsive element (RRE) and is currently being used to study the dimerization structure of HIV-1.

Membrane Structure and Function

The research goals in Dr. Robert Blumenthal's laboratory are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. The mode of action of the envelope protein of HIV, the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus are studied. Dr. Blumenthal and his colleagues have developed biophysical techniques to investigate the initial steps of viral envelope protein-mediated membrane fusion. Current studies of the quantitation of HIV-1 infection kinetics may provide new insights into mechanisms of AIDS progression and rates of HIV-1 infectivity and transmission. Other studies have demonstrated an essential requirement for calcium ions for cell fusion mediated

by the CD4-HIV-1 envelope glycoprotein interaction. Dr. Blumenthal's group has also studied the interactions of CD4 containing plasma membrane vesicles (CD4-PMV) with HIV-1 and HIV-1-Env protein-expressing cells and have shown that CD4-PMV were more effective at inhibiting syncytia formation than soluble CD4 alone, suggesting that CD4-PMV may have the potential to inactivate HIV-1.

In other studies the inability of animal cells expressing human CD4 to fuse with cells expressing the HIV-1 envelope glycoprotein was investigated using vaccinia vectors to express each protein. The data suggest that the block to fusion is not due to dominant inhibitory components in the animal cell; rather, that human cells contain additional component(s) which, when transferred to the CD4-bearing animal cells, confer the ability to undergo membrane fusion mediated by the HIV-1 envelope glycoprotein. Further studies have shown that the adhesion molecule LFA-1 does not play a crucial role in the early events of HIV-1 envelope glycoprotein-mediated cell membrane fusion, but rather contributes to the later events leading to giant cell formation.

LABORATORY OF PATHOLOGY

The Laboratory of Pathology, directed by Dr. Lance Liotta, is responsible for all the diagnostic services in anatomic pathology, surgical and postmortem pathology, neuropathology, ultrastructural pathology, cytopathology and electron microscopy for the Clinical Center of the NIH, and has research programs in various areas of experimental pathology. A fully accredited 4-year residency program in anatomic pathology is provided for 9 residents and 3 fellows.

Surgical Pathology

Dr. Maria Merino and her colleagues are 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. They are 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.

Postmortem Pathology

A comprehensive study of the pulmonary pathology of the acquired immune deficiency syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH. Molecular techniques are also being applied to the study of lung cancer.

Cytopathology

Dr. Diane Solomon has directed research efforts in cytopathology towards the application of immunocytochemistry for diagnosis. Lymphoid markers have been utilized to differentiate reactive processes from lymphoma, as well as to subtype lymphomas. Several monoclonal antibodies have been evaluated for specificity and sensitivity for the diagnosis of metastatic carcinoma in cavity fluids. Currently under investigation is the use of in situ hybridization as an ancillary diagnostic technique for cytology. Through a national consensus conference, Dr. Solomon's group has established the "Bethesda" system for standardizing the classification and reporting of cervical cytology smears.

Ultrastructural Pathology

Dr. Maria Tsokos conducts an active research program in the characterization of small round cell tumors of childhood. She has focused on the identification of markers and the development of techniques for the diagnosis, prognosis, and further histogenetic characterization of Ewing's sarcoma, primitive neuroectodermal tumors (PNET), and rhabdomyosarcoma. Immunohistochemical studies with various neural and muscle markers evaluated by Dr. Tsokos have shown the great value of muscle markers, and the limited value of neural markers in the differential diagnosis of neural from muscle tumors.

Dr. Tsokos has also initiated and participated in several studies involving p-glycoprotein expression by immunohistochemistry in pediatric tumors. Initial studies suggest that expression of p-glycoprotein is probably not responsible for treatment failures in this group of tumors.

Tumor Invasion and Metastases

A major focus of research activity is on elucidating the genetic changes associated with the metastatic phenotype. Understanding the mechanism of action of these genetic changes has led to new strategies for prognosis and therapy, some of which are currently being evaluated in clinical trials.

Dr. Mark Sobel is studying laminin receptors and their role in tumor cell metastasis. He has found a strong correlation between laminin receptor mRNA and protein levels and metastatic outcome for colon and breast cancer.

Dr. William Stetler-Stevenson is studying type IV collagenase, a metalloproteinase which specifically cleaves basement membrane type IV collagen and is augmented in metastatic tumors. Current evidence suggests that negative regulation of type IV collagenase may be mediated through TIMP-2, a novel human metalloproteinase inhibitor. TIMP-2 may function as a tumor suppressor protein by inhibiting metalloproteinase activity required for invasion. It may also arrest metastasis through inhibition of angiogenesis. Specific clinical applications of TIMP-2 could include the treatment of bone metastasis in breast and prostate cancer.

Dr. Patricia Steeg has obtained the full-length cDNA for NM23, a putative metastasis suppressor gene. Previous studies revealed a high degree of homology between the NM23 gene product and the awd protein involved in Drosophila development and morphogenesis, and to a gene product involved in the differentiation of Dictyostelium. Mutation or allele loss associated with NM23 may lead to a disordered state favoring malignant progression. Loss of NM23 expression in breast cancer is associated with a highly significant reduction in survival. Transfection of NM23 cDNA leading to augmented NM23 protein production abrogates metastasis. Recent studies indicate that the NM23 protein is an NDP kinase; the functional role of NM23 NDP kinase activity is under investigation. NM23 may provide a new approach to predicting the metastatic aggressiveness of an individual patient's tumor. Agents which modulate NM23 expression or function, or mimic its action, may have therapeutic potential.

Locomotion is a necessary component for tumor cell invasion. 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, and appears to stimulate a pertussis toxin-sensitive

motility response. Biochemical and histochemical studies of autotaxin are in progress. CAI, a novel signal transduction inhibitor which blocks tumor cell cytokine stimulated growth and motility, has been developed as a new cancer therapy approach. Clinical phase I trials of CAI for treatment of refractory cancers were initiated in March, 1992.

Hematopathology

Dr. Elaine Jaffe conducts a major program in diagnostic and experimental hematopathology. Her research focuses 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.

Dr. Maryalice Stetler-Stevenson has demonstrated that frequent relapse of follicular lymphoma is a consequence of clonal expansion of daughter cells derived from a common stem cell. To detect occult lymphoma, she has specifically amplified the joined bcl-2/JH DNA sequences created by the t(14;18) translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (PCR), she can detect 1 copy of bcl-2/JH, which is four orders of magnitude more sensitive than flow cytometry or Southern blot restriction analysis. Direct application to clinical samples has demonstrated lymphoma cells which were otherwise undetectable.

Dr. Mark Raffeld has completed a molecular analysis of small noncleaved cell lymphomas, further subclassified as sporadic Burkitt's type and non-Burkitt's. These studies confirm a molecular basis for the morphologic subclassification of small noncleaved cell lymphoma. In another collaborative project, Dr. Raffeld has completed a study of the molecular genetics of gastrointestinal non-Hodgkin's lymphomas. This study found a low incidence of bcl-1 and bcl-2 translocations, arguing for a different pathogenesis for gastrointestinal non-Hodgkin's lymphoma from that of node-based non-Hodgkin's lymphoma.

Gene Regulation

The goal of Dr. David Levens' research program is to define the biochemical mechanisms employed during the transcription, processing and translation of RNA and to identify pathology resulting from aberrant regulation. His work is focused in two areas: the transcriptional regulation of c-myc, and the trans-activation of the gibbon ape leukemia virus by a set of factors binding to AP1 sites from T-cells.

The Regulation of Lymphocyte Proliferation

Dr. Kathleen Kelly is investigating the consequences of mitogen-mediated signals to T cells. She has isolated over 60 novel cDNA clones that represent mRNA species induced by mitogens in human peripheral blood T cells. Several interesting functional classes of growth-regulated proteins have been revealed including a structurally unique class of tyrosine phosphatase, a novel GTP-binding protein associated with the endoplasmic reticulum, and a cell surface receptor with seven transmembrane regions that couple signal transduction through G proteins. These proteins are being studied with regard to biochemical properties and potential physiological function.

DERMATOLOGY BRANCH

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

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases

Dr. Katz' group has continued to study the immunological functions of cells of the epidermis. During the past year they have been investigating 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 (LC). Increased amounts of IL-1β mRNA from Langerhans cells can be detected as early as 15 min after hapten painting. Their studies suggest that IL-1β may mimic the effects of allergens on Langerhans cells on a molecular as well as a biological level. In addition, they have found that keratinocytes produce IL-10 protein both constitutively and after stimulation with some haptens. IL-10 abrogates Th1 proliferation and may play a critical role in the ultimate activation of Th2 cells. Further studies will determine its role in the T cell response to contact allergens. Studies of T cell activation by human Langerhans cells are continuing. Dr. Katz's group will investigate the immunological functions of LC in patients with AIDS.

Regulation of Cutaneous Accessory Cell Activity in Health and Disease

Dr. Mark Udey and his colleagues are involved in the study of costimulatory molecules important in Langerhans cell-T cell activation. Studies designed to identify important adhesion (or costimulatory) molecules on LC are ongoing. Dr. Udey's group has determined that LC express cadherins, that LC adhere to E-cadherin expressing cells in vitro and that anti-E cadherin MAb abrogates LC binding to keratinocytes. E-cadherin expression by LC reflects endogenous synthesis since they also contain E-cadherin mRNA. The role that cadherins play in LC biology and in the localization of other leukocytes in epithelia is under investigation.

Molecular Basis of Autoimmune Skin Diseases

Dr. John Stanley's laboratory studies autoantibody-mediated skin diseases in order to further understand not only the pathophysiology of these diseases, but also the structure and function of normal epidermis and the epidermal basement membrane zone. Pemphigus vulgaris (PV) is an acantholytic blistering disease wherein patients have autoantibodies to epidermal cell surface molecules. The full length coding sequence for PV antigen has been cloned. The deduced amino acid sequence of this antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules. PV patients have antibodies against the amino-terminal domain of this molecule, an area thought to be important in its adhesion function, and these antibodies can cause loss of adhesion of epidermal cells in an animal model of disease.

Therapy of Skin Cancer and Disorders of Keratinization

The goal of Dr. John DiGiovanna's group 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. His group is also actively collaborating with several groups to determine whether genetic linkage exists between certain heritable skin diseases and gene clusters.

METABOLISM BRANCH

The clinical research program of the Metabolism Branch, directed by Dr. Thomas Waldmann, is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. 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.

Molecular Analysis of Transacting Factors that Mediate Gene Expression

The control of gene expression in lymphoid development is the focus of research activity in Dr. Louis Staudt's laboratory. His studies have revealed that two transcription factors, Oct-2 and LEF-1, which are present at low or undetectable levels in the progenitor cells, are strongly induced upon differentiation to the lymphoid, but not the myeloid lineage. The molecular cloning of the lymphoid-restricted transcription factor, Oct-2, by Dr. Staudt helped to define the POU-domain transcription factor multigene family. Oct-2 and LEF-1 may play a role in commitment of a stem cell to the lymphoid lineage. The molecular cloning of a novel lymphoid-restricted gene, Ly-GDI, revealed that it encodes a protein bearing striking homology to a regulator of the ras-like G protein, rho, and may regulate events during lymphocyte activation. Another protein, JAW-1, has homology to the coiled-coil region of myosin and, surprisingly, resides in the endoplasmic reticulum. Both of these proteins reveal tissue-specific regulation of cellular processes that were previously thought to behave similarly in all cell types.

Somatic Gene Therapy for Human Genetic Disease

Dr. Michael Blaese's laboratory continues to focus on the development of gene therapy. He led the group which performed the first authorized use of gene transfer to treat human genetic disease when they infused autologous adenosine deaminase (ADA) gene-corrected T cells into a 4-year-old girl with ADA deficiency severe combined immunodeficiency disease (SCID). This girl, and a second ADA-deficient patient, have been treated 10-12 times over the past two years with such gene-corrected T cell infusions and are now showing signs of reconstituted immune reactivity. Dr. Blaese's laboratory has also developed a unique new approach to direct gene therapy of cancer using instillation of murine fibroblasts producing retroviral vectors directly into tumors in situ. Using vectors for herpes simplex thymidine kinase, he has shown a cure of brain tumors in rats following systemic administration of ganciclovir. Studies are in progress to use a similar approach to treat basic tumors in humans.

Mechanisms of Antigen-Presentation and T-Lymphocyte Recognition: Application to Vaccine Design

Dr. Jay Berzofsky's laboratory has studied the mechanisms by which T-cells recognize antigens presented on the surface of other cells in association with

major histocompatibility complex (MHC)-encoded molecules, and the application of these principles to the design of synthetic vaccines for AIDS and cancer. They have characterized a peptide fragment of the HIV-1 envelope protein recognized by $CD8^+$ cytotoxic T lymphocytes (CTL). Recently they identified a 10-residue HIV peptide that is a million times more active than the 15-residue HIV peptide previously identified in this laboratory. They have also showed that the class I molecule plays two roles in CTL activation, one to present the peptide and the other, presumably to interact with CD8; thus the MHC molecule binding CD8 does not have to be the same one presenting peptide. They have characterized the fine specificity of CTL for this peptide and shown that noncrossreactive CTL distinguish aliphatic from aromatic residues at a single position. This finding led to the discovery of a way to induce broadly crossreactive CTL against multiple variants of HIV by stimulation with a chimeric peptide. This peptide was also found to crossreact with a superficially unrelated peptide from the gp41 region of the envelope, even when presented by 4 different class I MHC molecules. This CTL site, which also is a target of neutralizing antibodies, has been coupled to other sites that stimulate T helper cells in mice and humans of multiple MHC types, and the resulting candidate synthetic vaccine has been found to induce extremely high titers of neutralizing antibodies in mice, as well as specific CTL killing of HIV-1 envelope expressing cells. A Phase I human immunotherapy trial with this construct is being planned. An early diagnostic test is also being developed. Dr. Berzofsky also showed that mice infected with schistosomiasis and then with a vaccinia virus recombinant expressing HIV envelope made reduced IL-2, interferon, and CTL responses specific for HIV envelope and had difficulty eliminating the virus. This may account in part for the rapid spread of AIDS in Africa, where such parasites are endemic. The same approach has been applied to cancer vaccines aimed at inducing CTL to mutant peptides corresponding to oncogene mutations (especially in p53 and ras that are common in many human cancers). Dr. Berzofsky's lab has succeeded in inducing CTL to a peptide corresponding to a p53 tumor suppressor gene mutation that will kill tumor targets expressing the mutant p53 gene. This demonstrates that such mutant oncogene products, although not expressed on the cell surface, can serve as targets of specific cancer immunotherapy.

The Multichain IL-2 Receptor: Molecular Characterization and Use as a Target for Immunotherapy

The research activity in the laboratory of Dr. Thomas Waldmann is focused on the development of a novel form of therapy, IL-2 receptor directed therapy. Normal resting T cells, B cells and monocytes do not express the IL-2 receptor. In contrast, large numbers of IL-2R are expressed by the abnormal cells in certain forms of leukemia, autoimmune diseases, and in patients rejecting allografts. In previous work, Dr. Waldmann identified two peptides that bind IL-2: the 55 KD protein IL2Rα chain reactive with the anti-Tac monoclonal antibody, and the 70/75 Kd IL-2Rß protein reactive with a monoclonal antibody termed Mikß1. He proposed a multichain model for the high affinity receptor in which both IL-2Rα- and IL-2Rß-binding proteins are associated in a receptor complex.

In related studies, Dr. David Nelson and his colleagues identified a soluble form of the p55 component (Tac protein) of the human interleukin-2 receptor in the supernatants of activated T-cells, B-cells, and monocytes in vitro and in the serum and urine of normal individuals in vivo. Elevated levels of soluble IL-2Rα were found in the sera of patients with ATL, hairy cell leukemia, the acquired immunodeficiency syndromes, and in patients with autoimmune diseases. Elevations

of serum IL-2Rα were also indicative of allograft rejection episodes in patients with liver and heart-lung transplants. Further studies have demonstrated that the measurement of soluble Tac protein in various body fluids is useful in monitoring certain neoplastic and immune-mediated events in vivo.

Dr. Waldmann initiated a therapeutic trial using unmodified anti-Tac monoclonal antibody in the treatment of patients with adult T-cell leukemia (ATL). There was no toxicity, and several of the patients studied underwent a remission. Although use of such murine antibodies is of value in the therapy of human diseases, their effectiveness is limited, since these rodent monoclonal antibodies often induce a human immune response. To circumvent this difficulty, genetically engineered antibody variants of anti-Tac were produced by combining the rodent genetic elements encoding the hypervariable regions with human, constant and framework region genes. Dr. Waldmann showed that the "humanized" version of the anti-Tac monoclonal antibody is dramatically less immunogenic than the parent mouse monoclonal. Furthermore, he showed that the "humanized" version of anti-Tac manifests an antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. With the lowered immunogenicity, improved pharmacokinetics, and a new effector function antibody-dependent cellular cytotoxicity it is hoped that there will be a substantial improvement in the therapeutic efficacy of this genetically engineered monoclonal antibody. Plans are underway to initiate therapeutic trials with "humanized" anti-Tac in patients with IL-2 receptor expressing malignancies. A clinical trial with this antibody has begun in patients with IL-2 receptor-expressing leukemia and lymphoma and in individuals undergoing graft versus host disease. In parallel studies a "humanized" version of Mikβ1 that blocks binding to the IL-2Rβ component has been generated by combining the complementarity determining regions of Mikβ1 with human immunoglobulin framework and constant regions. As with anti-Tac "humanized" Mikβ1 manifests antibody-dependent cellular cytotoxicity. Furthermore, the addition of "humanized" Mikβ1 complements the anti-IL-2Rα chain antibody anti-Tac in inhibiting IL-2 induced proliferation.

Drs. Waldmann and Nelson extended the clinical therapeutic implications of monoclonal antibodies by focusing on the use of these agents as carriers of cytotoxic agents. Here the goal is to maintain the specificity of the monoclonal antibody, while increasing its capacity to kill unwanted cells by coupling toxins or radionuclides to it. They developed cytotoxic agents wherein α- and β-emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. Examples include bismuth-212, an α-emitting radionuclide conjugated to anti-Tac. Yttrium-90, the β-emitting radionuclide, has also been chelated to anti-Tac. A dose escalation trial with Yttrium-labeled anti-Tac has been initiated for the treatment of HTLV-1-associated adult T-cell leukemia (ATL). Eleven of the 15 patients underwent a partial or complete remission following treatment.

EXPERIMENTAL IMMUNOLOGY BRANCH

The Experimental Immunology Branch, directed by Dr. Alfred Singer, carries out laboratory investigations in basic immunobiology with particular emphasis in 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; and transplantation biology. A flow cytometry laboratory supports multiple projects which involve quantitative, single-cell, multiparameter immunofluorescence analysis of cells prepared from a variety of tissues and cultured cells.

Lymphocyte Differentiation and Regulation

The molecular basis for low antigen receptor expression in developing $CD4^{-}CD8^{+}$ thymocytes has been studied extensively in Dr. Alfred Singer's laboratory. Previous studies have revealed that T-cell receptor (TCR) expression and function in developing thymocytes is actively regulated by CD4-mediated signals generated by the interaction of CD4 with Ia^{+} thymic epithelium. Release from intra-thymically generated inhibitory CD4 signals results in increased TCR expression, dephosphorylation of TCR-zeta chains, and improved TCR signaling. 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 intra-thymically generated CD4 signals mediated by p56 lck regulate the TCR degradation rate in $CD4^{-}CD8^{+}$ thymocytes. They have also examined the intra-thymic differentiation of functionally and phenotypically distinct T cell subsets, as well as their interaction with thymic epithelium.

The process of negative selection, by which potentially self-reactive T cells are deleted during development, has been analyzed in the laboratory of Dr. Richard Hodes to determine 1) the extent of T-cell receptor (TCR) Vβ deletions that occur in generation of the mature TCR repertoire, 2) the range of self-determinants that play a role in these TCR deletions, and 3) the relationship of these "deleting ligands" to the strong alloantigens that mediate high-frequency responses by mature T-cell populations. Their findings indicate that maintenance of tolerance to a variety of self-determinants results in substantial deletions in the available TCR Vβ repertoire. The self-determinants that function as ligands for Vβ-specific T-cell deletions generally represent the products of non-MHC-encoded genes in association with MHC gene products. Ligands responsible for deletion of Vβ11- and Vβ12-expressing T-cells were shown to represent a previously uncharacterized Mls "superantigen" capable of inducing a strong response by allogeneic T-cells. Thus, the set of Mls superantigens appear to play a critical role as self-determinants in shaping the TCR repertoire by negative selection. Mapping of the non-MHC genes contributing to Vβ-specific deleting ligands has in each case identified an endogenous mouse mammary tumor (MMTV) provirus associated with deletion. Additional studies have demonstrated that efficient TCR negative selection is thymus dependent.

Exogenous retroviruses were analyzed for their influences on T cell repertoire. Milk-borne MMTV induced Vβ14 deletion only in strains of mice bearing natural or transgenic I-E class II major histocompatibility complex (MHC) product. 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.

Although Vβ-specific negative selection mediated by endogenous superantigens provides 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. An analysis of the coexpression of specific Vα/Vβ pairs by individual 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.

Dr. Stephen Shaw's laboratory has been systematically analyzing heterogeneity among subsets of human T-cells and the functional capacities of those subsets. Continuing analysis has emphasized β1 and β7 integrins and CD31, because of their known importance in adhesion and adhesion regulation. His lab has identified a unique monoclonal antibody Act-1 and structurally characterized the molecule recognized as the α4β7 integrin where α4β7 is selectively expressed at high levels on unique subset of CD4 memory cells. α4β7-high CD4 cells are phenotypically unique among memory cells in many respects and by multiple criteria are inferred to be gut homing T cells.

Dr. Gene Shearer's group is investigating human T helper cell (TH) function in asymptomatic HIV-infected (HIV+) individuals; HIV-exposed individuals who exhibit no evidence of infection; and patients with systemic lupus erythematosus (SLE). It was found that both HIV+ individuals and SLE patients exhibit a spectrum of TH functional defects which are predictive for disease progression and are associated with changes in the profiles of immunoregulatory cytokine production. A significant number of HIV-exposed, seronegative individuals from high risk groups were found to exhibit in vitro TH function to synthetic peptides of HIV gp120. Studies in these at-risk groups and newborn infants to HIV+ mothers suggest that HIV-specific TH function is protective against HIV infection and/or progression to AIDS.

Cell Biology of Immune Responses

The expression and function of cell adhesion molecules by B-cells has been analyzed by Dr. Richard Hodes' laboratory. IL5 induces B cell proliferation and immunoglobulin (Ig) secretion and results in appearance of a phenotypically novel B cell population which expresses high density of CD44 and low densities of B220 (CD45) and Ia. This B cell sub-population mediates nearly all of the proliferative and Ig secretary activity of IL5-activated B cells. Additional evidence suggests a potential role for CD44 in regulating trafficking of activated B cells in vivo. Thus, functional states of the CD44 molecule may exist, perhaps reflecting differences in conformation or cytoskeletal association.

Dr. Shaw's laboratory has been identifying and characterizing the functions of cell surface molecules which participate in T cell recognition and adhesion, and the functional roles of multiple adhesion pathways in T cell interactions with endothelium. A new aspect of these studies is analysis of in vitro expanded TIL (tumor infiltrating lymphocytes). Phenotypic analysis demonstrates that the integrins on TIL are already in a functionally activated state and show little further augmentation in binding when activated by various agents.

Signal Transduction

Studies in Dr. Alan Weissman's laboratory have focused on the zeta subunit of the T cell antigen receptor (TCR), which undergoes tyrosine phosphorylation in response to receptor engagement and is important in TCR-mediated signal transduction. The molecular mechanisms responsible for the regulation of tyrosine phosphorylation of the TCR are under investigation. Current data suggest that G proteins play a synergistic role in enhancing phosphorylation induced by antireceptor antibody. In the course of the analysis of human, rat, and murine zeta immunoblots, Dr. Weissman's laboratory noted previously unappreciated degrees of heterogeneity. Recent findings indicate that zeta is ubiquitinated. Subsequent experiments have found that CD3 δ is also

ubiquitinated. Further analysis suggests that phosphorylated zeta can also serve as a substrate for ubiquitination. These ubiquitinated subunits are not isolated intermediates in a pathway to degradation, but are components of apparently intact TCRs, and occur in all T cell types that have been analyzed.

The zeta subunit of the T cell antigen receptor is a limiting component in receptor assembly and is required for the targeting of the T cell antigen receptor to the cell surface. Dr. Weissman's group characterized the gene encoding the human zeta subunit. The intron/exon organization of the zeta gene and the sites of transcription initiation have been determined. Studies are underway to determine the promotor and enhancer elements of the zeta gene.

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. 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. 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 shown that class I gene expression is cyclically regulated in response to hormonal stimulation.

Lymphocyte Effector Function

In studies on the mechanism of lymphocyte-mediated cytotoxicity, Dr. Pierre Henkart's laboratory has established the role of cytotoxic lymphocyte granzymes in triggering "apoptotic" damage to target cells, including DNA fragmentation. Their approach was transfection of a mast cell tumor line with combinations of the lymphocyte granule components cytolysin and granzyme A. Transfectants expressing only the membrane pore-forming cytolysin killed targets without DNA breakdown, while transfectants expressing only granzyme A were not cytotoxic. Transfectants expressing both showed tumor target cytotoxicity with accompanying DNA breakdown. Injection of several different well-characterized proteases into tumor cells triggers apoptotic cell death, with DNA breakdown preceding lysis, membrane blebbing and cell shrinkage. These data further support the granule exocytosis model for lymphocyte-mediated cytotoxicity.

Developmental Biology

Dr. Michael Kuehn's laboratory has used insertional mutagenesis to identify genes that have important roles in mouse embryogenesis. Mouse embryonic stem cells, infected with a retroviral vector in order to induce insertional mutations, have been injected into embryos, resulting in transgenic mice carrying multiple independent mutations in the germ line. As a result of analyzing two transgenic strains, Dr. Kuehn's laboratory identified 27 proviral insertions, five of which were associated with recessive mutations that disrupt embryonic development. One of the mutant genes is a lethal recessive that causes early death of the embryo. The gene, named hyperplastic ectoderm (*hec*), is located on chromosome 10 and is being cloned for molecular analysis. Techniques have been developed in Dr. Kuehn's laboratory for improving the efficiency of following the inheritance patterns of large numbers of proviruses intentionally inserted into the germ

lines of transgenic mice. This will enable a 20-40 fold increase in the number of insertions that can be studied in each transgenic mouse strain.

Transplantation Biology

Dr. Ronald Gress and his colleagues have undertaken studies 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. They have demonstrated that a cloned CTL population is sufficient to reject an allogeneic marrow graft, and that 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. 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 and also enhanced engraftment of MHC-mismatched, T cell depleted marrow in vivo. Experiments with transgenic mice have demonstrated that clonal deletion is the mechanism by which veto cells mediate suppression of the CTL response.

The elimination of cells expressing T cell surface markers from marrow is of interest both in allogeneic and 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. These approaches have been used to develop clinical protocols to assess the feasibility of utilizing allogeneic HLA-mismatched, T cell depleted marrow and autologous marrow purged of malignant T cells in the treatment of aggressive hematolymphopoietic malignancies.

The data from murine studies are consistent with the possibility that residual T cells in the infused marrow play a central role in the generation of subsequent T cell populations in the recipient. The functional capacities of regenerated T cell populations following T cell depleted marrow transplantation has also been studied. It was found that the human T helper cell response to xenogenic MHC encoded antigens expressed by stimulating murine cell populations requires reprocessing of the stimulating murine antigens and presentation in association with human class II gene products, and that is due in part to a lack of murine antigen presenting cell activation.

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY

A major focus of research activity in the Laboratory of Tumor Immunology and Biology, directed by Dr. Jeffrey Schlom, has been the generation of monoclonal antibodies to tumor-associated antigens and the development of these monoclonal antibodies for the diagnosis and treatment of cancer. During the past year Dr. Schlom and his colleagues have concentrated on the development of a novel approach to cancer vaccines; the use of vaccinia virus tumor antigen constructs for the specific active immunotherapy of cancer.

Active Immunotherapy to Human Carcinoma-Associated Antigens

Active specific immunotherapy is a new and potentially nontoxic approach for cancer therapy. Carcinoembryonic antigen (CEA) is a 180,000 dalton oncofetal glycoprotein expressed on most gastrointestinal carcinomas and several other human carcinoma types including many breast cancers. CEA is generally considered

to be weakly immunogenic in humans; however, recent studies have shown that the copresentation of CEA with a strong immunogen (such as vaccinia virus) offers a way to induce anti-CEA responses for tumor immunotherapy. Dr. Schlom and his colleagues have developed a CEA-vaccinia construct which can be recognized by an anti-CEA monoclonal antibody. Immunization of mice with the recombinant vaccinia virus resulted in a humoral immune response against CEA. Pilot studies demonstrated that the administration of the recombinant vaccinia virus was able to greatly reduce the growth of tumors in mice. The use of this new recombinant CEA vaccinia virus may provide a new approach in the specific active immunotherapy of human gastrointestinal cancer, breast, and other CEA expressing carcinoma types.

Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens.

Dr. Schlom's laboratory has had a long-standing interest in the generation, characterization, and utilization of monoclonal antibodies (MAbs) directed against antigens associated with human carcinomas. MAb B72.3 can selectively bind the "pancarcinoma" tumor-associated glycoprotein TAG-72 and has been shown to selectively target a range of carcinomas in clinical trials involving over 1000 patients. Studies have been conducted to characterize a series of "second generation" MAbs to the TAG-72 antigen. Some of these second generation CC MAbs, such as CC83 and CC49, have a higher affinity constant to TAG-72 than B72.3 and may be better suited for clinical applications. CC49 has been shown to efficiently target human colon carcinoma xenografts and is currently being evaluated in both diagnostic and therapeutic clinical trials. ^{177}Lutetium (^{177}Lu) conjugated CC49 was shown to delay the growth of established LS-174T human colon carcinomas in athymic mice at a single dose of 50 µCi. ^{177}Lu-CC49 is now being considered as a potential novel therapeutic for human carcinoma.

To date, radiolabeled B72.3 has been given to over 1,000 patients in tumor-targeting studies carried out in numerous institutions, with similar findings of approximately 70-80% tumor targeting observed. A phase I therapy trial involving intraperitoneal administration of ^{131}I-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity has been conducted. Extremely good localization of tumor lesions in the abdomen was detected. The use of a recombinant/chimeric MAbs has been conducted. Clinical trials involving the use of second and third generation Mab-isotope conjugate are in progress.

Mammary Tumorigenesis in Inbred and Feral Mice

MMTV appears to induce tumors by acting as an insertional mutagen that leads to the activation of expression of a previously silent cellular gene or the rearrangement of a normally expressed gene (int genes). Dr. Robert Callahan's group has found a dichotomy in the frequency with which the wnt-1 gene is activated in tumors arising within preneoplastic hyperplastic outgrowth lines (6%) and those arising in situ (52%) in the mammary glands of C3H breeders, suggesting that wnt-1 activation provides a proliferative advantage to transformed mammary epithelial cells in intact C3H mammary glands. They have determined the nucleotide sequence of the 2.3 kb RNA species whose expression is activated by MMTV insertion in the int 3 locus in mammary tumors. It encodes a 57kD protein which is 50% identical to the intracellular portion of the neurogenic Drosophila notch gene product. A transgenic mouse strain has been established containing activated int-3 as the transgene. Focal and often multiple poorly differentiated mammary and salivary gland adenocarcinomas occur

in 100% of the transgenic mice between 2 and 7 months of age. Significantly, mammary glands were arrested in development and were lactation deficient in all female int-3 mice.

The Identification and Characterization of Human Genes Associated with Neoplasia

Dr. Callahan's laboratory has undertaken an ongoing program that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association with the patient's history, characteristics of the tumor, and the patient's prognosis. In previous studies they have found a loss of heterogeneity (LOH) on chromosomes 1p, 1q, 3p, 11p, 13q, 17p, 17q, and 18q. Current results demonstrate LOH of the c-met proto-oncogene on chromosome 7q in 40.5% of the tumor DNAs. This genetic alteration was not associated with any of the standard prognostic features of breast cancer; however, patients having tumors with LOH on chromosome 7q had a significantly shorter disease-free interval and overall survival. A separate panel of 96 primary breast tumors was evaluated for their proliferative index by their ability to incorporate BrdU in culture. A significant association was found between those tumors having an elevated BrdU labeling index and LOH at the pYNZ22.1 locus on chromosome 17p. In contrast, no association was found between the tumor BrdU labeling index and LOH at the more telemeric locus p144D6 on chromosome 17p. Studies are in progress to determine whether the p53 tumor suppressor gene is a target for LOH on chromosome 17p, or whether p53 mutations are linked to the proliferative index on the tumor.

SUMMARY REPORT

LABORATORY OF GENETICS, DCBDC, NCI

October 1, 1991 through September 30, 1992

In 1991-92 the Laboratory of Genetics organized the 10th Mechanisms in B-Cell Neoplasia workshop that was attended by approximately 150 participants. This field continues to be a focus of current interest as several outstanding forms of B-cell tumors including multiple myeloma continue to increase in incidence in the US for unexplained reasons. The workshop stimulated many interactions and collaborations with workers outside the NCI. The contract with Hazleton Laboratories to maintain our conventional mouse colony and carry out tumor induction experiments was renewed in the spring of 1992 for an additional 5 years. As in previous years, 1991-1992 has been a year in which there has been considerable turnover in the non-permanent staff.

Progress has been made in the search for genes that determine susceptibility (S) and resistance (R) to the induction of plasmacytomas by chronic peritoneal irritants such as pristane. The model system on which much of the work focuses are the S/R genes that determine resistance in DBA/2 and susceptibility in BALB/cAn. Strong preliminary evidence that DBA/2 R genes were located on chromosome 4 has been confirmed. Mapping of the genes has been made possible by Beverly Mock who has identified four new allelomorphic genes and screened and assembled potential genes described in other laboratories. The current evidence indicates there are two DBA/2 Pct-R genes on the distal end of chromosome 4. Beverly Mock also has developed an assay for the susceptibility phenotype and shown that the S genes are also on chromosome 4. Much of this analysis involves the use of a panel of BAL/cAn.DBA chromosome-4 congenic strains that have been developed in the LG. Several of these strains have a strong partial resistance phenotype. Work continues on the identification of the physiological properties of the Pct-S/R genes. We pursue the hypothesis that some of these genes increase the probability that B-cells in BALB/cAn mice are more prone to developing chromosomal rearrangements affecting the c-myc gene. We are encouraged by preliminary evidence developed in collaboration with V. Bohr that the C.D2-chromosome-4 congenics the have partial resistance are also the DBA/2 efficient DNA repair phenoptype.

Work continues on the biology of plasmacytomagenesis. Emily Shacter has developed data that show that both pristane and plastic disc peritoneal irritation is associated with great increases in local IL-6 production. Francis Wiener has found that his BALB/cAn.Rb6.15 congenic strain is hypersusceptible to plasmacytoma induction by pristane and A-MuLV. Independently, Stuart Rudikoff and Francis Wiener have developed strong evidence that the BALB/c B-cell and not the microenvironment is the site of action of the S/R genes by reconstitution experiments in SCID mice. Dr. Rudikoff's lab is exploring the mechanism of immunological reconstition of SCID mice using selective populations of cells and found differences in potentialities of peripheral lymph node and Peyer's patch lymphocytes to reconstitute the immne systems of these animals.

The incidence of plasmacytomas has declined in our BALB/cAnPt colony which could be due to genetic drift or environmental factors. Intraperitoneal immunization with heterologous red blood cells restors the incidence. Sandra Smith-Gill is exploring the role of viral antigens on plasmacytomagenesis in pathogen-free BALB/cAn mice.

Frederic Mushinski and Konrad Huppi continue to search for a function of the complex 200-300kb Pvt-1 locus, the target of the T(6;15) translocation in mice and the T(2;8) and T8;22) translocations in man. Although a protein product has not been identified, increased transcription in normal cells is associated with mitogenic stimulation.

Linda Wolff and Katherine Nason-Burchenal using a probe to the retroviral-myb insertion site that activates c-myb in murine pro-monocytic leukemias have detected by PCR methodology the presence of the insertions as soon as 3 weeks after viral infection. These 'mutant' cells represent a pre-neoplastic change in myeloid cells, and this work opens the possibility for studying in detail the pathogenesis of promonocytic leukemia in mice.

Wendy Davidson has characterized the T-cell populations associated with the massive lymphoproliferation in lpr/lpr and gld/gld mice and is exploring the hypothesis that lymphoproliferation results from defective regulation of the pool of primed T and B cells. She is studying the behavior of the various T cell populations arising in lpr/lpr and gld/gld mice in SCID mice.

Sandra Smith-Gill continues analysis of monclonal antibody-lysozyme interations. The X-ray structure of a HyHEL-5 Fab complexed with a site directed mutant of HEL which reduced the binding affinity by 10^3 has been solved and refined. Pursuit of these model systems will hopefully establish principles that can be applied to the design of antibody molecules to proteins.

Hayden Coon has demonstrated that the olfactory mammalian neuroblasts that have been successfully cultured do, in fact, carry the differentiation markers of olfactory cells. This model culture system will be of great value to the study of the growth and differentiation of blast cells. He continues to develop culture systems for growing cell types that have been difficult to establish *in vitro*.

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

PROJECT NUMBER
Z01 CB 05552-23 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mammalian cellular genetics and cell culture

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

P.I.: H.G. Coon Research Biologist LG, NCI

COOPERATING UNITS (if any)
Assoc. Prof. L. Buck, Department of Neurobiology, Harvard Med. School, Boston, MA; Prof. F.S. Ambesi-Impiombato, Asso. Prof. F. Curcio, Istituto di Patologia, Clinica e Sperimentale, Udine, Italy

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5

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

B

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

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

Major Findings:

This year we have pressed ahead with our effort to grow normal human cell strains from the endocrine pancreas. We have found that it is possible to grow beta-cells slowly (7-10 day population doubling time) and other cells from the islets of Langerhans more rapidly. We have renewed our efforts to culture human olfactory neurons and have demonstrated that cells from a minute scratch biopsy can be grown for at least 20-30 doublings before the division rate slows excessively.

We have established methods in our lab for the identification of differentiated cells from islets using both RIA and immunohistochemistry.

We have found, in collaboration with Dr. Adi Gazdar, that our media and culture techniques are capable of culturing human lung tumor cells that had proven impossible to grow in the past. Particularly interesting was the fact that with a simple modification of our medium we could grow lung tumor cells without contamination by mesothelial or fibroblast normal cells that usually confuse cultured biopsy material. These observations suggest potentially helpful methodologies to do individualized testing of chemotherapeutic drugs and regimes on cultures prepared from tumor biopsies.

Finally, we have continued our long standing interest in the mechanism of coding olfactory signals by olfactory neurons. We have entered into a collaboration with Dr. Linda Buck (Department of Neurobiology, Harvard Medical School, 200 Longwood Avenue, Boston, MA). Our findings thus far are only that the experiments (described below) are feasible.

In summary, we again present more results than could normally be expected given the poor support given to our work. Ultimately, it is true that "minimally supported" research usually yields minimal results. Happily, our fortunes will change after a change of venue for the PI when he retires from the NIH sometime after August 16, 1992.

The new (never before described in these reports) project is that undertaken with Dr. Linda Buck at Harvard, and this project is chosen for description in somewhat greater detail below.

Nosidomas and Olfactory Coding:

In the spring of 1991, Linda Buck and Richard Axel reported the identification of a family of genes from rat mRNA that fulfill our expectations for the elusive vertebrate olfactory odorant receptors. This family has more than the anticipated number of members (currently estimated at >200) 50 or more of which have been cloned and partially sequenced. One of the principal barriers to an adequate understanding of vertebrate olfaction has been the fact that none of these receptors had been isolated. We did not know how many receptors there might be or how many receptors were expressed by a given sensory neuron. Our group had shown that our clones from rat were differentially sensitive to chemical test odorants, but until the receptors could be identified, of course, they could not be enumerated or identified or compared from one cell type to another. Does each cell type produce a single receptor? or 10 or even 100

receptors? Now that Linda Buck has identified this large gene family, the fun will begin. To study this problem, we want clonal cell lines that can be compared for which receptors they express. We are working with mice which suggests that hybrid cell lines, or transformed cell lines could be suitable. We have chosen to start with hybrids between N-18TG2, John Minna's 6TG-resistant mutant of the C-1300 mouse neuroblastoma (the same line that he and Coon and Nirenberg made hybrids with mouse and rat brain cells back in the early 1970's). Those hybrids expressed neuronal phenotypes and some of them have proven useful for many years - expressing, for example, opiate receptors among others. Dr. Buck and I hope that such hybrids will continue to express receptor phenotypes when they are made between sensory neurons and N-18s. As of this writing we have made such hybrids and are starting to analyze which of the olfactory receptors each of them expresses. Coon had proposed making such hybrids in the rat system 4 or 5 years ago - however, without the identified and cloned olfactory receptor probes there was no feasible way to characterize them. Now that Linda Buck has gotten such probes, the way is clear to use our hybrid technology to produce 'nosidomas' much as Millstein and Kohler have shown up with a similar problem, the immunocytes and their 'hybridomas'. We have made two series of hybrids between the cells of the olfactory mucosa of 5 week old mice and the selectable (HGPRT-, HAT-sensitive N-18 6TG-2). These hybrids can be tested by 1) in situ hybridization of individual or pools of probes made from members of the olfactory receptor family or 2) by PCR using known sequences as primers and cDNA from mRNA prepared from individual or pooled hybrids. Ultimately, the goal of determining how receptors are expressed among the sensory neurons and even how the selection of a particular receptor population is made. At that point the fundamental mystery, analogous to the combinatorics of light and heavy chains of immunoglobulin, of 'coding' in the olfactory epithelium, should become unraveled. It is good that our lab could play a part in this culmination of the olfactory problem.

Publications:

Wolozin B, Sunderland T, Zheng B, Resau J, Dufy B, Barker J, Swerdlow R, Coon H. Continuous culture of neuronal cells from adult human olfactory epithelium. J Mol Neurosci 1992;3:137-46.

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

PROJECT NUMBER
Z01 CB 05553-23 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

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

COOPERATING UNITS (if any)
Dr. Kevin Holmes, NIAID, NIH; Dr. A. Anderson, US Army Institute of Infectious Disease, Frederick, MD

LAB/BRANCH
Laboratory of Genetics

SECTION
Molecular Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5	3	2

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

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

BALB/c mice are highly susceptible (S) to plasmacytoma (PCT) induction protocols while DBA/2 mice are resistant (R). Previously we examined the cellular basis for this phenomenon by transferring BALB/c or DBA/2 bone marrow (BM) to SCID mice and injecting the chimeras with either pristane, or the retrovirus J3V1 containing deregulated myc and raf oncogenes. Using these induction protocols we determined that DBA resistance could not be overcome by the susceptible SCID microenvironment suggesting that R/S, in this system, is dictated by the donor BM.. Recent studies of SCID mice reconstituted with peripheral, mature BALB/c lymphocytes either alone, or co-transferred with DBA/2 BM suggest the following: 1.) Mature BALB/c B cells from spleen, lymph node or Peyer's patches are equally susceptible to tumor induction; 2.) DBA/2 cells do not suppress BALB/c PCT development; and 3.) BALB/c lymphocytes can not supply DBA/2 B cells with the extra-cellular signals required for PCT development. Thus, DBA/2 resistance and BALB/c susceptibility to PCT induction are traits inherent to the respective B cells. II. SCID mice have also been used to study normal lymphocyte development and life-span. Previously, we determined that adoptive transfer of Peyer's patch cells to SCID mice resulted in normal reconstitution of all lymphoid tissues except the thymus in which there appeared only single positive CD4 and CD8 mature T cells. We have expanded these studies to determine the duration of this reconstitution and the reconstituting potential of peripheral (PLN) and mesenteric (MLN) lymph node cells. Results indicate that: 1.) PLN cells reconstitute peripheral lymphoid organs, but are less effective at repopulation of the gut mucosal tissues than Peyer's patch cells; 3.) The thymii of PLN and MLN reconstituted SCID mice contain donor CD4 and CD8 single positive mature T cells; and 4.) PP, MLN and PLN repopulation persists for greater than 6 months. These results demonstrate that the lifespan of mature lymphocytes is longer than previously suggested. Moreover, the homing properties of these cells in SCID mice indicate that mucosal and peripheral lymphocytes differ in their ablility to modulate homing receptor expression and consequently their pattern of lymphoid tissue reconstitution.

Major Findings:

I. The prototypic susceptible and resistant mouse strains for plasmacytoma induction are BALB/c and DBA/2, respectively. Classical genetic analyses involving these strains have demonstrated that susceptibility and resistance are regulated by multiple genes on different chromosomes. While studies are underway in the Laboratory of Genetics to further define these genetic loci, little is known about susceptibility and resistance at the cellular level. The complex nature of plasmacytomagenesis allows for the possibility that susceptibility and resistance could be attributable to inherent differences in B-, T-, or accessory/stromal cell populations which presumably contribute to the granulomatous microenvironment in which plasmacytomas arise. In order to assess the relative contribution of each cell type to susceptibility and resistance we have used the SCID mouse as an adoptive host for a variety of BALB/c and DBA/2 cells. Since SCID mice were derived from susceptible (i.e. C.B17) stocks, and display a number of phenotypic and genotypic differences compared with DBA/2 and BALB/c they allow direct assessment of the cellular components of plasmacytomagenesis.

Previously we have demonstrated that injection of pristane and the retrovirus J3V1 (containing the myc and raf oncogenes) results in a tumor incidence of 65% (25/40) in BALB/c bone marrow reconstituted SCID mice. Analysis of these tumors using flow cytometric, Southern blot, and immunoglobulin secretion assays demonstrated that 40% (10/25) of these tumors were plasmacytomas and 60% (15/25) were of myeloid origin. In contrast, similar treatment of DBA/2 bone marrow reconstituted mice yielded only myeloid tumors with an incidence of 44% (17/39). More recently, we have analyzed DBA/2 and BALB/c bone marrow reconstituted mice subjected to the classical plasmacytoma induction protocol of 3 injections (0.5ml/injection) of pristane only. At one year post treatment none of the reconstituted mice showed gross signs of tumor development. However, analyses of granulomatous tissue from these animals using the "foci counting" method developed by Dr. Michael Potter revealed that 60% (9/15) of SCID's receiving BALB/c bone marrow harbored plasmacytomas, while no (0/11) tumors were identified in mice receiving DBA/2 bone marrow. These results indicate that a susceptible (SCID) microenvironment is not sufficient to allow development of plasmacytomas among genetically resistant (DBA/2) cells using either classical or virus-dependent tumor induction protocols.

Although the failure of DBA/2 reconstituted mice to develop plasmacytomas is not due to a deficient microenvironment, it remains possible that DBA/2 bone marrow derived cells are inhibitory to tumor development, or alternatively, DBA/2 mice lack a population of lymphoid cells necessary for plasmacytoma development. To test these possibilities we reconstituted SCID mice with a mixture of mature, peripheral BALB/c lymphocytes taken from spleen, lymph node or Peyer's Patches, and DBA/2 BM. Following a virus-dependent tumor induction protocol, these mice displayed a tumor incidence of 60% (49/82) of which 80% (34/49) were plasmacytomas of BALB/c origin. No (0/49) DBA/2 plasmacytomas were observed. The development of BALB/c plasmacytomas in these double chimeras indicates that all cells requisite for plasmacytomagenesis are present and that DBA/2 bone marrow-derived cells are not inhibitory to this process. Moreover, the absence of DBA/2 plasmacytomas can not be attributed to a lack of an appropriate lymphocytic/stromal microenvironment. Thus, resistance to plasmacytomagenesis reflects properties inherent to the DBA/2 B cell.

The experiments described above raise several questions regarding plasmacytomagenesis. First, although no DBA/2 plasmacytomas were observed, a number

of DBA/2-derived myeloid tumors arose in chimeric mice suggesting that resistance is specifically associated with DBA/2 B cells. The mechanism(s) specifically precluding DBA/2 B cell transformation remain undefined. Second, it is well documented that de-regulation of the myc oncogene by chromosomal translocation is a critical event in plasmacytomagenesis and possibly in human Burkitt's lymphoma. Our use of the J3V1 tumor induction protocol suggests that resistance is not solely attributable to an inability of DBA B cells to de-regulate myc, since integration of the J3V1 retrovirus results in the constitutive expression of the myc and raf oncogenes. Finally, the ability to generate plasmacytomas from mature, peripheral BALB/c lymphocytes taken from spleen, lymph node or Peyer's Patches demonstrates that potential tumor target cells are located throughout the mouse. The molecular sequence of events leading to their transformation remains unclear, however, the above described studies clearly identify a critical cellular component in determining susceptibility and resistance.

II. Plasmacytomas are induced in BALB/cAnPt mice by the intraperitoneal introduction of plastic materials, mineral oils, or alkanes such as pristane. Such treatment results in the formation of a chronic granulomatous tissue on the peritoneal surfaces which is the site of the developing plasmacytoma. One of the hallmarks of primary plasmacytomas is that ascitic tumor cells may not be successfully transplanted when introduced into the peritoneal cavities of normal, syngeneic BALB/cAnPt mice. |Such tumors can only be transplanted into mice which previously received (i.p.) a small volume (0.5ml) of pristane. However, the dependence of primary plasmacytomas on the oil induced microenvironment is usually lost after several transplant generations, indicating that microenvironment and growth requirements of early developing plasmacytomas differ from those of long term transplanted tumors. Consistent with this view has been the inability to grow primary plasmacytomas in vitro, while, in contrast, many serially transplanted plasmacytomas have been adapted to tissue culture.

In order to study the growth requirements and early events of granuloma dependent tumors, we have developed a system in which primary plasmacytomas can be expanded in vitro using an autologous feeder layer of stromal cells from the initial site of plasmacytomagenesis. The early neoplastic lines established in this manner are dependent on physical contact with the stromal layer for growth and survival. The stromal cells provide at least two stimuli for the plasma cells, one being IL-6 ant the second, of unknown nature, resulting from direct physical interaction which cannot be replaced by soluble factors such as rIL-6 or stromal cell supernatants. The interaction between plasma and stromal cells appears to be mediated, at least in part, by CD44 as either hyaluronidase or mab to CD44 inhibits adhesion by 70-80%. The plasma cell lines obtained in this manner have been passaged for as long as 20 months without the loss of their in vivo dependence on the oil induced microenvironment. The ability to now grow primary tumors in culture and maintain their in a "primary" state provides a unique model system for detailed analyses of early events in tumor progression involving neoplastic cells completely dependent on physical contact with a stromal feeder layer for survival and expansion.

III. Lymphocyte development, recirculation, and dissemination throughout the host clearly involve multiple cell types which necessarily interact via direct cell-cell contact, as well as, soluble factors. The mechanism(s) regulating these processes must allow for the appropriate seeding of all immune system tissues including mucosal tissues, spleen and an extensive network of peripheral lymph nodes. Since each of these structures displays a unique cellular composition, architecture and effector function it has been suggested that

organ-specific homing molecules and their respective ligands may be associated with this phenomenon. For example, Peyer's patches, which line the gastrointestinal tract, are considered prototypic structures within the mucosal immune system. These structures are characterized by a number of unique features. First, PP cells preferentially and rapidly home to the lamina propria and PP following adoptive transfer to compatible hosts. Second, entrance of circulating lymphocytes to PP appears restricted and may be dependent, at least in part, on expression of VLA4LPAM-1 determinants on the cell surface and the corresponding ligand on PP high endothelial venules. Third, a high frequency of IgA precursors is found among PP B cells. Fourth, freshly isolated, or cloned T cells obtained from PP preferentially enhance switching of IgM to IgA and differentiation of B cells.

To understand the mechanism(s) resulting in the sequestration of particular cell types within various lymphoid organs, this laboratory has assessed the ability of specific cells to populate the lymphoid system of immuno-incompetent SCID mice. Previously, we have demonstrated that adoptive transfer of Peyer's patch cells to SCID mice resulted in normal reconstitution of all lymphoid tissues except the thymus in which there appeared only single positive CD4 and CD8 mature T cells. We have expanded these studies to address questions of lymphocyte lifespan and the reconstituting potential of other restricted lymphocytic populations such as peripheral (PLN) and mesenteric (MLN) lymph node cells. FACS analyses of both PLN and MLN show no significant differences between the populations. Lymphocytes from these tissues are predominantly T cells (64%), of which virtually all are TCR alpha/beta+ expressing either CD4 (77%) or CD8 (33%). B cells comprise 38% of total lymph node cells, the majority (60%) being IgM+. Interestingly, although several reports have identified MEL-14 as a LN-specific homing receptor, only 30% of lymph node B cells and 59% of T cells express this putative LN homing receptor.

The results obtained after reconstitution of SCID mice with MLN or PLN cells are as follows: 1.) Serum Ig profiles in PLN and MLN reconstituted SCID mice are qualitatively and quantitatively normal within one month of transfer. 2.) Within 27 days of transfer T cells account for 10-18% of all splenic cells. Both CD4+ and CD8+ cells are present in the normal ratio of approximately 3:1 and the total number of T cells per spleen is about 40% of that found in normal DBA/2 spleen. 3.) By day 111 splenic T cells have increase to 15-24% which, although representing an essentially normal T percentage, is still only approximately half of the total number of T cells found in normal DBA/2 spleen. 4.) During the 111 days following reconstitution, splenic B cells increase gradually from 3-6% on day 27 to 12-19% on day 111. 5.) FACS analyses of thymii from both PLN and MLN reconstituted SCID mice demonstrates the presence of single positive CD4 and CD8 T cells which all express TCR alpha/beta. No donor derived double positive cells were detected. 6.) Histological analyses of lymphoid organs in reconstituted mice shows that in contrast to PP repopulated SCID's, PLN chimeras do not efficiently repopulate the lamina propria of the gut. 7.) As with PP cells, PLN and MLN reconstitution persisted for greater than 5 months.

Taken together these results raise several questions regarding the mechanisms regulating the lifespan, recirculation and capacity for self-renewal among mature, peripheral lymphocytes. First, the longterm survival and proliferation of PP, MLN, and PLN cells in SCID mice suggests that either the absence of established regulatory networks within SCID mice allows for continuing lymphocyte expansion, or that previous studies have underestimated lymphocyte half-lives. Second, the observed reconstitution suggests that, if lymphocytes 'home' in an organ specific manner controlled by receptor

expression, such a process is dynamic in that receptors can be modulated on and off the cell surface, or lymphocytes simultaneously express multiple homing receptors. This process is not without limitations since PLN derived cells appear less capable of effectively repopulating the gut of SCID mice. Third, it is surprising that mature, peripheral lymphocytes, or a subpopulation thereof, have retained the capacity for self-renewal. The precise cells responsible for the observed reconstitution remains undefined but may involve a small population of precursor cells distinct from those found in either bone marrow or thymus. Alternatively, the availability of "space" in the SCID mouse may allow naive lymphocytes to continue expanding until the appropriate regulatory networks are established. Finally, the observed reconstitution may be due to expansion of long-lived memory cells, the repertoire of which is sufficiently large to yield an apparently normal lymphocytic repopulation.

The SCID mouse is clearly an invaluable model for the study of immune system reconstitution, lymphocyte trafficking and acquired immune function resulting from transfer of specific lymphoid populations. It should now be possible, with a combination of magnetic bead pre-selection and cell sorting, to isolate specific sub-populations and determine both their in vivo trafficking patterns and proliferative potential.

Publications:

Rudikoff S. Principles of tumor immunity: biology of antibody mediated responses. In: DeVita VT, Hellman S, Rosenberg SA, eds. Biologic therapy of cancer: principles and practice. 1991.

Shin S-U, DePinho R, Zack D, Rudikoff S, Scharff MD. The instability of immunoglobulin genes in the S107 cell line. Somat Cell Mol Genet 1991; 17:259-76.

Rudikoff S, Fitch WM, Heller M. Exon specific gene correction (conversion) during short evolutionary periods: homogenization in a two gene family encoding the beta-chain constant-region of the T-lymphocyte antigen receptor. Mol Biol Evol 1992;9:14-26.

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

PROJECT NUMBER
Z01 CB 05596-23 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

P.I.:	M. Potter	Chief, Lab. of Genetics	LG, NCI
	E.B. Mushinski	Bio. Lab. Technician	LG, NCI
	B. Mock	Staff Fellow	LG, NCI
	E. Shacter	Expert	LG, NCI
	V. Bohr	Sr. Investigator	LMP, NCI
	S. Janz	Visiting Associate	LG, NCI

COOPERATING UNITS (if any)
Dr. F. Wiener, Karolinska Inst., Stockholm, Sweden; Dr. K. Marcu, SUNY, Stony Brook, NY; J. Shaughnessy, E.J. Beecham, Univ. of MD Graduate Students

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 6	PROFESSIONAL: 5	OTHER: 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 major project in the laboratory is to determine pathogenetic mechanisms involved in the development of paraffin oil (pristane) induced plasmacytomas in BALB/c mice. BALB/cAn mice are highly susceptible to developing these tumors while most other strains are resistant. Over 95% of plasmacytomas induced by pristane have chromosomal translocation [rcpt(12;15), rcpt(6;15)] involving directly or indirectly the c-*myc* locus on Chr 15. The specific problems on which we are working are: 1) to locate and characterize genes that determine resistance and susceptibility to plasmacytoma induction by peritoneal irritants such as pristane, 2) define environmental factors (antigens, diet,infections) that affect the rate of plasmacytoma formation, and 3) to identify critical mutations that cooperate with c-*myc* dysregulation in the neoplastic transformation of plasma cells. Substantial progress has now been made with finding plasmacytoma resistance genes of DBA/2 origin. Using a series of BALB/c.DBA/2 congenic strains two genes on Chr 4 have been localized by linkage to *Pnd* and *Lgm-1* that determine partial resistance to plasmacytoma induction by pristane. The functions of these genes are not known, but phenotypically they inhibit the formation and progression of pre-neoplastic plasmacytic foci and plasmacytomas. This phenotype can be detected at day 150 by use of a focus enumeration assay. We are searching for other correlative phenotypes using various DNA repair assays. The progressive isolation of our mouse colony has been associated with a decrease in the incidence of pristane induced plasmacytomas. We have found that immunization with heterologous red blood cells restores the incidence to the 60% level that prevailed 10 years ago when the colony was not so clean. In collaborative studies with K. Marcu we are studying the plasmacytomagenic properties of transforming retroviruses that contain *Ha-ras* + *p53*, *IL-6*, *BCL-2* or *E1a*. All of these viruses rapidly induce plasmacytomas, but the effectiveness is quite variable.

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

Environmental Factors: Plasmacytoma induction is influenced by antigenic stimulation. Pathogen free BALB/cAn mice are refractory to plasmacytoma induction by pristane. Further, the BALB/cAnPt mice in our colony at Hazleton laboratories have begun showing a reduction in plasmacytoma incidence from the expected yeild at 300 days of 60% to as low as 32% in some experiments. We attribute this downward fluctuation to the isolation of our colony from other mice and cleanliness of our colony. Another possibility is that variations in diet may also be a factor. To standardize the yield of plasmacytomas, mice have been immunized with repeated i.p. injections of heterologous (sheep, horse and chicken) red blood cells. At 300 days 32% of control BALB/cAn mice had developed plasmacytomas, while 58% of the mice that received 7 i.p. injections of rbc beginning at day -10 and continuing through day 123 developed plasmacytomas. Experiments are now in progress to determine the effects of immunization on the incidence of plasmacytomas induced by pristane in the partially resistant C.D2-chr-4 congenic mice.

We have completed a histological study on the pathogenesis of plastic disc induced plasmacytomas. The plastic discs induce a variable amount of fibrotic like tissue on some peritoneal surfaces, including the disc. Plasmacytomas do not appear to develop in this reactive tissue (the counterpart of the oil granuloma). Developing plasmacytomas were found, however, in the omentum in association with milk spots. This finding suggests a relation to the milky spots and the $CD.5^{+}$ B cells.

In collaborative experiments with Beverly Mock the genetic mapping of the distal third Chr 4 linkage map has now been extended with a number of new allelomorphic markers that distinguish BALB/c from DBA/2. A series of C.D2-chromosome-4 congenics have been constructed that have different DBA/2 chromatin segments. Plasmacytoma induction and foci counting experiments on these mice are in progress. An important finding thus far is that C.D2-Fv-1n/n and C.D2-TolFam3 which carry non-overlapping segments of DBA/2 chromatin and are both partially resistant to plasmacytoma induction, thus indicating there are two Pct-R genes on Chr 4 that determine partial resistance.

Plasmacytoma induction experiments require 300 days for completion. However, plasmacytomas develop gradually from day 80, and this progression can be assessed by determining the number of plasmacytic foci in the mesenteries between days 120 and 150. Experiments to determine the effect of Pct-R genes on focus formation have indicated differences can be detected at day 150. In experiments done three years ago before we encountered the problem with a reduction in incidence in our BALB/cAn controls, differences could be determined between days 100 and 120. In a focus assay study the mesenteries are removed from 20 mice and the number of foci are determined. This assay should then accelerate the genetic studies on localizing the *Pct-R* genes. In addition, sequential focus studies can be used to study the progression of pre-neoplastic lesions to more advanced neoplasia.

The current working hypothesis of focus development is that these lesions arise in oil granuloma tissue by seeding of cells from the peritoneal space. Morphologically, it appears that many of the foci have characterisitics that suggest a metastatic rather than an *in situ* origin. Further, we have been able to show that the presence of a very few plasma cells in the peritoneal space (ascites) is associated with multiple focus formation, suggesting that focus cells are able to detach and seed onto other locations. This opens up the possibility of trying to isolate these early cells.

Immunophenotyping of foci (with E.B. Mushinski) with a variety of antisera that are specific for Ig heavy and light chain classes have been carried out on 62 mice. Of these 20 (32%) were bi- or triclonals. In many the second clone was minor, but in 5 mice there were more balanced mixtures. A frequent variant was the appearence of a clone that failed to secrete a heavy chain; these might be explained as a variant of an original clone. New experiments with idiotypic sera such as antisera specific for V-regioms.

Genetic Studies: The major effort of our genetic studies is to determine the physiological function of the resistance and susceptibility genes. One working hypothesis is that these genes influence the rate of formation of illegitimate chromosomal rearrangements. Such a phenotype might be associated with a difference in the efficiency of DNA repair. Following initial studies with Katherine Sanford we found that there were strain differences in the efficiency of repair of DNA damage induced by X-irradition. The work has been extended in collaborative studies with Vilhelm Bohr utilizing UV induced damage and an excision repair assay. This assay is carried out with specific regions of DNA and hence is a preferential repair assay. Five genes have been studied: *DHFR*, *Myc*, *Abl Pvt-1* and *Sa*. No differences have been detected with *DHFR* and *Abl*, but there are strong differences in the repair of the 5′ end of *C-myc*, *Pvt-1* and *Sa*. LPS blasts (B-cells) were used as the target tissue. These studies have now been extended to use the the DBA/2 congenic strains that have shown partial resistance to plasmacytoma induction. To carry out this work Mr. Gary Jones has joined the laboratory and Edward Beecham has been working with him to establish this complex assay in our laboratory.

Search for Cooperating Oncogenic Mutation: We continue our collaboartive study with Ken Marcu on testing combinations of oncogenes for their ability to rapidly induce plasmacytomas in pristane conditioned mice. Dr. Marcu has constructed a number of retroviral vectors that carry various combinations of oncogenes. We have previously found that *myc* + *Ha-ras*, *raf-1*, *A-raf* or *v-Abl* are effective plasmacytomagens. Currently under study are a series of non-myc containing viruses that contain *V-Ha-ras* + *p53*, *E1a*, *IL-6* or *Bcl-2*. Among these the *v-Ha-ras* + *E1a* is the most plasmacytomagenic. All of the other viruses have produced some accelerated plasmacytomas. Francis Wiener is karyotyping these plasmacytomas.

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

Tissue origin and characterization of the MPC-precursor cell. The main objective is to define which tissues contain cells that can be transformed into plasmacytomas (PCs). For this purpose cells were transferred into SCID mice or X-irradiated mice (radiochimeras). In many of the experiments the cells were infected in vitro with A-MuLV prior to transfer. The recipient mice were injected i.p. with a single 0.5 ml dose of pristane. A-MuLV accelerates plasmacytomagenesis in pristane conditioned mice. The mice used in these experiments were BALB/cAn, BALB/cAn.Rb6.15, BALB/cAn.Rb8.12 and BALBA/cAn.Rb4.12.

SCID experiments. The main results are as follows: a) The spleen contains MPC precursors from which donor plasmacytomas did develop; b) similar to the experiments with bone marrow the PCs that developed in SCID mice repopulated with spleen cells carried the T(12;15) translocation.

Radiochimeric experiments. Reconstitution of lethally irradiated mice i.v. route allows the transfer of a relatively low number of PC precursor cells. Consequently, the use of the cell transfer approach in the radiochimeric system has limited applicability. To circumvent this restraint experiments were performed to assess whether reconstitution via i.p. injection of larger number of cells could be accomplished. Lethally irradiated BALB/c mice were reconstituted i.p. with large numbers of BALB/c6.15 derived spleen and bone marrow cells. The reconstituted mice were typed for the presence of donor cells and their survival was followed approximately 4 months. The results of the transfer experiments in both the SCID and radiochimeric system convincingly demonstrated that both the bone marrow and the spleen contain B-cells from which plasmacytomas could develop. The results of the transfer experiments support this notion.

Plasmacytomas that arose in homozygous BALB/cRb6:15 mice induced by pristane + A-MuLV carry in a high number the variant 6;15 translocation in the form of inv(Rb6;15) translocation chromosome. The question that we are asking is what would be the ratio between the variant T(6;15) and invRb(6;15) of PCs induced in (BALB/c6;15;15xBALB/c)F1 mice? The reason for asking it is the topographical relationship between the myc and Ig-kappa genes in BALBa/cRb6.15 cells. If centromeres are attached to the nuclear scaffold, both arms of the Rb5.15 chromosome would be now in the same sector of the nucleus. This would increase the probability that these DNA segments could close enough to permit an illegitimate exchange (translocation). The preliminary conclusions that could be drawn from this experiment are as folows: i) The lack of T(12;15) and T(15;16) translocations suggests that in the BALB/c6.15 strain the MYC and KAPPA genes located on Rb6.15 are more accessible to illegitimate recombination than those located on separated chromosomes 15 and 6.

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

The inheritance of susceptibility to pristane-induced plasmacytomagenesis has been examined in 750 backcross progeny generated between BALB/cAnPt females (*Pct-1*^{s}) and male F1 hybrids (*Pct-1*^{r}) between BALB/c and DBA/2. Preliminary RFLP analyses of a small subset of these susceptible backcross progeny for a series of 3-4 markers/chromosome had indicated linkage of one of the genes (*Pct-1*) controlling susceptibility to mouse Chromosome 4. During the past year, RFLP analyses of a series of 18 markers distributed across the entire length of Chromosome 4 were performed on a series of 70 susceptible and 130 resistant backcross progeny. These analyses position Pct-1 in the distal part of mouse Chr 4 near the markers *D4Lgm3*, *D4Mit13*, *Gt-10* and *Tnfr-1*. We are continuing to isolate random DNA markers from Chr 4 in an effort to generate additional probes for analysis. This region of mouse Chr 4 is homologous with human Chr 1p and will, therefore, be of interest to examine in multiple myeloma samples.

An alternate method of plasmacytoma induction has involved the inoculation of retroviral vectors carrying differing combinations of oncogenes in addition to small and single doses of pristane. Tumors usually arise within 30-60 days post-inoculation under this induction protocol; the normal latency period for pristane-induced tumors averages between 220-260 days. Previous experiments with the RIM retroviral vector, which carries ras and myc sequences, the AM vector, which carries abl and myc sequences, and the J3V1 vector, which carries raf and myc sequences, have shown that BALB/c mice are susceptible and DBA/2 mice are resistant to tumor induction under these protocols; in addition, the genetic control appears to be modulated by a single gene in the RIM system. Backcross experiments have not been performed with the AM or J3V1 vectors. In an effort to determine the chromosomal location of the susceptibility/resistance gene in the RIM system, and to determine if the same gene may influence tumor development in the AM and J3V1 systems, a series of bilineal C.D2 congenic strains of mice, harboring DBA/2 donor genes from a variety of different chromosomes, are being evaluated for their S/R phenotypes following inoculation with pristane plus each of these retroviral vectors.

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

Aims:

The goal of the research is to elucidate the role of chronic inflammation in promoting B cell neoplasia and to determine whether inherited responses to inflammatory factors may determine genetic susceptibility. By employing mouse plasmacytomagenesis as a model, we are identifying specific products of the chronically inflamed environment that could mediate tumor development and growth. These include potential initiating agents (mutagens) such as endogenously-generated reactive oxygen intermediates, and tumor promoters such as cytokine growth factors. Inflammatory exudates from pristane-primed mice have been examined for the presence of interleukin 6 (IL-6), which is an essential growth factor for plasmacytoma cells *in vitro*, and for oxidized

proteins which may serve as markers for endogenous neutrophil activity *in vivo*.

Major Findings:

(1) Studies on IL-6. Plasmacytomas require IL-6 in order to proliferate in culture. We have found that injection of the mineral oil pristane into the peritoneal cavities of BALB/c mice leads to development of chronic high levels of IL-6 that are probably sufficient to support the growth of transformed plasma cells *in vivo*. A correlation is observed between the development of ascites tumors and the presence of elevated IL-6 at late times (~300 da) post-pristane. Administration of the non-steroidal anti-inflammatory drug indomethacin reduces the levels of IL-6 achieved *in vivo* and also inhibits IL-6 production by pristane-elicited macrophages *in vitro*. The results provide evidence that pristane induces an inflammatory cytokine that can act as a tumor promoter *in vivo*. They also suggest that indomethacin may inhibit plasmacytomagenesis in part by limiting proliferation of abnormal plasma cells that are dependent upon Il-6 to grow.

The possibility was examined that differential regulation of IL-6 levels *in vivo* might constitute a mechanism for susceptibility and resistance to plasmacytomagenesis. When we measured the levels of IL-6 that develop in different strains of mice in response to pristane, we found that BALB/c mice maintain the highest levels compared to resistant strains [DBA/2, (CxD2)F1 hybrids, SCID, C3H]. Moreover, macrophages isolated from the different strains showed different capacities to secrete IL-6 in response to exogenous stimuli (e.g., lipopolysaccharide). It is not known whether the differences are sufficient to contribute to genetic susceptibility; i.e., whether the levels in resistant mice are inadequate to support the growth of neoplastic plasma cells *in vivo*. Still, the finding of inbred strain differences suggests that the genes that regulate IL-6 during chronic inflammation may be investigated by comparing activities in high- and low-responder mice.

(2) Studies on neutrophil activity in vivo. Neutrophils constitute roughly half of the cells in the peritoneal cavities of pristane-primed mice throughout the latent period of plasmacytomagenesis. It is hypothesized that these cells can serve as a source of endogenous carcinogens because of their capacity to secrete reactive oxygen intermediates that can damage the DNA of neighboring cells. We have demonstrated some unique characteristics of neutrophil-induced DNA damage that support this view.

However, our studies on the role of neutrophils in plasmacytomagenesis have been restricted to experiments carried out with isolated cells *in vitro*. In recent experiments, our goal has been to establish whether pristane-elicited neutrophils are stimulated to produce oxidants *in vivo*. We are looking for manifestations of neutrophil-induced oxidative stress by assaying for oxidized macromolecules in the inflammatory exudates. In collaboration with Dr. R. Levine (Laboratory of Biochemistry, NHLBI) we discovered that oxidized proteins (protein carbonyl groups) accumulate in the peritoneal cavities of pristane-treated mice. The oxidized moieties segregate into a high molecular weight (>200 kD) protein fraction in gel filtration HPLC, suggesting that only selected proteins become oxidized during the inflammation

in vivo. In addition to providing evidence for endogenous neutrophil activity, the results raise the possibility that the oxidized proteins may have an activity that plays a role in the specific development of B cell tumors; e.g., by serving as endogenous antigens that drive B cell proliferation, stimulating macrophage accessory cell function, etc. Experiments are underway to identify the oxidized proteins and to investigate their biological function.

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

(1) Phagocyte-mediated DNA damage. During the last three years I have been working in the research group of Dr. Emily Shacter on diverse topics in the potential role of DNA damage and repair in plasmacytomagenesis in BALB/c mice. Our investigations were primarily focused on phagocyte-mediated DNA damage in B lymphocytes. For the first time, we provided direct experimental evidence that activated neutrophils induce unscheduled DNA synthesis in murine B cells. The results lay the foundation for future investigations into the cellular mechanisms for repair of oxidatively-modified DNA. In the context of that work, we also attempted to understand better the still unknown mechanisms of action of the plasmacytomagenic agent pristane (2,6,10,14-tetramethyl-pentadecane). We developed a novel solubilization method of pristane (US patent pending) in order to test the compound under more controlled conditions *in vitro*. Basic studies were carried out on the interaction of pristane with model membranes. The results provided experimental support for the hypothesis that an oxidation product of pristane may cause DNA damage *in vivo*. For the latter studies we employed a short-term bacterial genotoxicity assay (SOS chromotest) that is also being used to screen genotoxic activities of murine phagocytes.

(2) Mutagenesis in the 5′ flanking region of c-*myc*. Although the above mentioned research projects provided interesting avenues for continuing studies, I have currently turned to a molecular biological project. Recently, an inefficient DNA repair phenotype in the 5′ regulatory sequences of the proto-oncogene c-*myc* has been observed in plasmacytoma susceptible BALB/cAnPt mice but not in plasmacytoma resistant DBA/2N mice. This finding implies a relationship between differential repair of c-*myc* and genetic predisposition to plasmacytoma development and, more generally, between gene-specific DNA repair and B cell transformation. Because of the central role of c-*myc* in various forms of lymphomagenesis in man and animals and the interesting variety of mutations of c-*myc* (i.e., base exchanges, chromosomal translocations and viral insertions), the confirmation of this interrelationship is of great importance. Consequently, I am currently trying to verify the differential repair of c-*myc* with an independent method that is based on quantitative polymerase chain reaction. I have developed a PCR method to amplify a 1.8 kb genomic sequence from the 5′ regulatory portion of c-*myc* utilizing nested pairs of primers. Further experiments will determine if this sequence is long enough to measure differences in DNA repair after treatment with genotoxic agents. Furthermore, extensive work needs to be done to optimize the detection of the amplification products as well as to determine the sensitivity, the reproducibility and the intra- and interassay variation

of these experiments. Also, mutagens that are presumably more relevant than UV-radiation to murine plasmacytomagenesis should be tested.

The experience gained from these attempts to amplify relatively long *c-myc* sequences from genomic DNA have proved to be valuable for establishing a protocol to detect plasmacytoma specific translocations between *c-myc* and IgA sequences. So far, I have tested a considerable number of primers with different PCR protocols and also cloned PCR products into plasmid vectors. I have been focusing on primers derived from BALB/c consensus sequences of S_α but probably run in a dead end because of the repetitiveness of these sequences and the presumable existence of inverted repeats. For that reason I am currently sequencing the intervening sequence between S_α and $C_\alpha 1$ in order to get better primers. I want to point out that John Shaughnessy provided substantial help for the latter experiments. We would like to apply PCR of T(12;15) to a number of exciting problems in plasmacytomagenesis such as location, time, frequency, reversibility, and *in vitro* occurrence of these translocations.

Publications:

Modi WS, Pollock DD, Mock BA, Banner C, Renauld J-C, Van Snick J. Regional localization of the human glutaminase (GLS) and interleukin-9 (IL9) genes by in situ hybridization. Cytogenet Cell Genet 1991;57:114-6.

Janz S, Brede O, Muller J. The reaction of pristane (2,6,10,14-tetramethylpentadecane) with radiolytically generated reactive oxygen intermediates results in a stable genotoxic compound as assessed by the SOS chromotest. Carcinogenesis 1991;12:1241-6.

Janz S, Shacter E. A new method for delivering alkanes to mammalian cells: preparation and preliminary characterization of an inclusion complex between beta-cyclodextrin and pristane (2,6,10,14-tetramethylpentadecane). Toxicology 1991;69:301-15.

Shacter E, Arzadon GK. Elevation of IL-6 in plasma cell tumorigenesis. AIDS Res Hum Retroviruses 1992; in press.

Shacter E, Arzadon GK, Williams J. Elevation of IL-6 in response to a chronic inflammatory stimulus in mice: Inhibition by indomethacin. Blood 1992; in press.

Janz S, Shacter E. Molecular encapsulation and delivery of alkanes to living mammalian cells for risk assessment and pharmaceutical applications. US Patent Application. June 28, 1991, Docket #1173-292P.

Chin H, Kozak CA, Kim H-L, Mock B, McBride OW. A brain L-type calcium channel $\alpha 1$ subunit gene (CCHL1A2) maps to mouse chromosome 14 and human chromosome 3. Genomics 1991;11:914-9.

Mock BA, Neumann PE, Eppig JT, Huppi K. Mouse chromosome 15. Mammal Genome 1991;1:S241-68.

Blank R, Eppig J, Fiedorek F, Friedman JM, Huppi K, Jackson I, Mock B. Mouse chromosome 4. Mammal Genome 1991;1:S51-78.

Watson ML, D'Eustachio P, Mock BA, Steinberg AD, Morse HC, Oakley RJ, Howard TA, Rochelle JM, Seldin MF. Definition of a molecular genetic linkage map of mouse chromosome 1 using an interspecific cross segregating for the gld generalized autoimmunity mutation. Mammal Genome 1992;2:158-71.

Chin H, Mock B, Kim H-L, Kim H, Kozak CA. The gene for the dihydropyridine-sensitive calcium channel alpha 2 subunit (CCHL2A) maps to the proximal region of mouse chromosome 5. Genomics, in press.

Hanley-Hyde J, Mushinski JF, Sadofsky M, Huppi K, Krall M, Kozak CA, Mock B. Expression of murine cyclin B1 mRNAs and genetic mapping of related genomic sequences. Genomics, in press.

Gawrisch K, Janz S. The uptake of pristane (2,6,10,14-tetramethylpentadecane) into phospholipid bilayers as assessed by NMR, DSC, and tritiated pristane methods. BBA 1991;1070:409-18.

Obrams GI, Potter M. eds. Epidemiology and biology of multiple myeloma. Berlin: Springer-Verlag, 1991.

Largaespada DA, Kaehler DA, Mischak H, Weissinger EM, Potter M, Mushinski JF, Risser R. A retrovirus that expresses v-abl and c-myc oncogenes rapidly induces plasmacytomas. Oncogene 1992;7:811-9.

Potter M. Perspectives on the origins of multiple myeloma and plasmacytomas in mice. Hematol Oncol Clin North Am 1992;6:211-23.

Byrd LG, McDonald AH, Gold LG, Potter M. Specific pathogen-free BALB/cAn mice are refractory to plasmacytoma induction by pristane. J Immunol 1991;147:3632-7.

Byrd L, Grossmann M, Potter M, Shen-Ong GLC. Chronic multifocal osteomyelitis, a new recessive mutation on chromosome 18 of the mouse. Genomics 1991;11:794-8.

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

PROJECT NUMBER
Z01 CB 08727-15 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Organization and control of genetic material in plasmacytomas

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

P.I.:	J.F. Mushinski	Section Chief	LG, NCI
	J. Hanley-Hyde	Senior Staff Fellow	LG, NCI
	K. Huppi	Expert	LG, NCI
	H. Mischak	Visiting Fellow	LG, NCI
	E, Weissinger	Visiting Fellow	LG, NCI

COOPERATING UNITS (if any)
I. Magrath, K. Bhatia, PB, NCI; H.C. Morse, III, LVD, NIAID; J.H. Pierce, CIP, NCI; M. Kazienetz, P. Blumberg, CCTP, NCI; F.D. Finkelman, Dept. of Med., USUHS; W. Kolch, Goedeke A.G., Freiburg, Germany

LAB/BRANCH
Laboratory of Genetics

SECTION
Molecular Genetics

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
10	5	5

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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.)

Our research goal is to understand the genes whose altered structure or expression play critical roles in malignancy, autoimmune diseases and normal differentiation. We are concentrating on the expression of a group of these "oncogenes": *abl*, *bcl*-2, *bcl*-3 and *myc*, as well as the potential oncogenes, Pvt-1, Protein Kinases C (PKC) and cyclins, in mouse hemopoietic tumors. Deregulated expression of *myc* secondary to chromosome translocation has been shown to be one essential component of the genetic alterations involved in oil-induced i.p. plasmacytomas in BALB/c mice. Some of these *myc*-activating translocations occur 200-300 kb 3' of c-*myc* in a region called Pvt-1. We have shown that this locus is transcribed at very low levels in normal cells but in much higher amounts in some plasmacytomas and in certain B lymphocytic cell lines in which both c-*myc* and Pvt-1 genes are amplified. Normal splenic lymphocytes and NIH3T3 cells increase their expression of Pvt-1 following mitogenic stimulation, suggesting that Pvt-1 is a new member of the "early response" genes, although its function remains unknown. Human and mouse homologies have been detected, indicating evolutionary conservation and implying a function that is essential to normal growth or development.

The ABL-MYC retrovirus, expressing v-*abl* and c-*myc*, rapidly induces intraperitoneal plasmacytomas in BALB/c and other strains of mice that are resistant to pristane-induced plasmacytomagenesis. If the mice are preimmunized, 50% develop tumors producing antibody specifically directed against the immunogen. This has already proved to be a useful alternative to hybridoma technology for generating monoclonal antibodies to a variety of antigens.

Seven isozymes of the PKC family have been shown to be expressed in a cell-type specific fashion in hemopoietic cells and cell lines. These serine-threonine kinases have been shown to change the phenotype of NIH3T3 cells when overexpressed by transfected expression vectors, and immunofluorescent studies with isotype-specific antibodies indicate that each isotype translocate to different subcellular locations after its kinase activity has been activated by phorbol ester. Similarly, overexpression of certain PKCs in mouse myeloid cell lines imparts to these cells the ability to respond to phorbol ester stimulation by differentiating into macrophages.

I. Expression of c-*myc* in Plasmacytomas:

1. The IgG1 secreting plasmacytoma, ABPC 60, harbors a novel reciprocal t(12;15) translocation in which the chromosome 15 breakpoint occurred 240 kb 3' of c-*myc* and resulted in the fusion of the *Pvt*-1 major breakpoint cluster to the IgH Sα locus. Whereas all previously described t(12;15) translocations involve a break 5' of c-*myc* and result in the head-to-head juxtaposition of c-*myc* with an IgH switch region, both conventional and pulsed field gel electrophoresis restriction mapping suggest that the ABPC 60 t(12;15) resulted in a paradoxical head-to-tail juxtapostion of c-*myc* to Cα, a phenomeneon inconsistent with the germline chromosomal orientations of these loci. We propose that the unexpected orientation of the t(12;15) was caused by an inversion in chromosome 12 in which the inversion breakpoints occurred 3' of the Cα gene and 5' of the J_H gene cluster followed by the t(12;15) translocation.

2. Two plasmacytomas (ABPC 22 and RFPC 2782) lack chromosomal translocations but have a deregulated c-*myc* due to a "head to head" retroviral integration immediately 5' of the c-*myc* gene, thereby implicating enhancer insertion as the mechanism of c-*myc* deregulation in these tumors. The steady-state c-*myc* mRNA levels in ABPC 22 are two-fold less than those seen in RFPC 2782 and lower than any plasmacytoma studied to date. Sequence analysis of the LTRs of the c-*myc*-associated proviruses showed that only the ABPC 22 provirus lacked one of the transcription enhancer's direct repeats, therefore implying that enhancer effectiveness in B-cell tumors in *vivo* may require both direct repeats. The PCTs described here represent the first examples of murine B-cell tumors that harbor retroviral insertions in the c-*myc* gene, and these results support the notion that c-*myc* rearrangement, whether by chromosomal translocation or retroviral integration, is essential in the genesis of mouse plasmacytomas.

II. Molecular Organization and Function of Mouse Pvt-1:

The Pvt-1 region was originally defined as a cluster of chromosome translocation breakpoints or retroviral integration sites associated with B-cell or T-cell neoplasms. Up-regulated expression of c-myc appears to be one important consequence of these genetic rearrangements, even though Pvt-1 is located about 260 kb distal to c-*myc*. The expression pattern of Pvt-1 is similar, in many ways, to that of c-*myc* which is found on the same chromosome. This pair of oncogenes is also co-amplified and jointly over-expressed in several mouse B lymphomas, which leads us to hypothesize that c-*myc* and Pvt-1 may make up a single functional genetic unit, e.g. a *megagene*. The isolation of human and mouse cDNAs that correspond to Pvt-1 sequences have provided evidence for the existence of a Pvt-1 gene product. The isolation of a Pvt-1 protein, however, has been hindered by the inability to find long open reading frames (ORFs) within Pvt-1 cDNA sequences. In a search for longer ORFs, we have analyzed Pvt-1-positive cDNAs from two mouse sources; a B-cell lymphoma in which c-*myc* and Pvt-1 are co-amplified, and a plasmacytoma with a t(6;15) translocation that interrupts the Pvt-1 locus. We have now identified a 57-bp stretch of DNA which is consistently found at the amino-terminus of alternatively spliced transcripts of mouse Pvt-1. This region, designated Pvt-1a, contains two AUG initiation codons, resides in exon 1 and comprises part of the largest ORF (140 aa) established to date in Pvt-1. Alternative splicing of Pvt-1 transcripts is evident 5' and 3' of the Pvt-1a segment in these cDNAs, and a detailed map of the Pvt-1 locus is being constructed from a large series of murine and human cDNA and genomic clones .

III. Overexpressed c-myc and v-abl in the ABL-MYC retrovirus rapidly induced plasmacytomas.

1. When mouse cells are infected *in vivo* or *in vitro* with the ABL-MYC retrovirus, plasmacytomas are obtained in 100% of the animals within only a few weeks. This rapidity of induction suggests that the combination of over-expressed c-*myc* and v-*abl* may be sufficient to fully transform mature B cells. Plasmacytomas arise even earlier, however, in BALB/c nude mice, which suggests that T-cell surveillance plays a role in suppressing plasmacytomagenesis. Nonetheless, T-cell depletion was not sufficient to render C3H mice sensitive to plasma cell tumor induction by ABL-MYC.

2. If mice are immunized with any of a variety of antigens before plasmacytomas are induced with ABL-MYC, about half of the mice develop plasmacytomas that secrete antibodies to the immunogen. These are frequently monoclonal, but, if not, they can be made monoclonal by *in vitro* cultivation and cloning. We have been successful in producing useful monoclonal antibodies to a growing number of antigens in this way.

IV. Somatic mutation in mouse plasmacytomas:

Several lines of evidence suggest that up-regulation of c-*myc* is not the only genetic lesion associated with mouse plasmacytomagenesis. However, we have have found no evidence of *ras* mutations in a survey of mouse plasmacytomas. We and others have shown, by single-stranded conformational polymorphism (SSCP) of PCR-amplified DNA, that p53 is frequently mutated in human Burkitt's lymphomas and B-ALL (L3), both of which also exhibit up-regulated c-*myc* gene expression. Despite this correlation between up-regulated c-*myc* and p53 mutation in human tumors, we have found no p53 mutations in mouse plasmacytomas. This result may indicate that the mouse is significantly less susceptible to this form of carcinogenic point mutation than man, or it may be that the comparisons were made between tumors of different degrees of maturation. Since plasmacytomas represent a later stage in B-cell development than Burkitt's lymphomas or B-ALL, mouse neoplasms of less mature B cells may contain p53 mutations, suggesting that wild-type p53 promotes lymphocyte differentiation.. To test this hypothesis, we have begun a search for SSCP evidence of p53 mutations among a panel of mouse pre-B cell and T cell tumors of different degrees of maturation.

V. Protein Kinase C:

We have cloned complete cDNAs of PKC–δ, -ε, –ζ and –η from mouse cDNA libraries and sequenced these cDNAs in order to learn the sequence of these important molecules in the mouse. PKC–δ is the most abundantly expressed PKC isoform in hemopoietic cells, and its structure has been conserved in rat, mouse and man. The published structure of rat PKC–ζ is very unusual for an isoform of PKC inasmuch as it lacks 1 of the 2 cysteine-rich zinc fingers that characterize all of the other PKC isoforms and it is not activated by phorbol esters. Our data show that mouse PKC–ζ also has this structure and unresponsiveness to phorbol esters, which indicates that it has been conserved in evolution and undoubtedly has some unique role among the family of PKC isozymes.

Functional Differences among PKC Isoforms:

Several different expression vectors for all 7 isoforms have been constructed, and the protein is being overexpressed in baculovirus-infected insect cells to accumulate large amounts of each mouse PKC isoform for structural and functional studies and for production of isoform-specific antibodies. When PKC-containing mammalian expression vectors are introduced into mouse promonocytic 32D cells, only overexpressed PKC–α and –δ, but not PKC–β, -ε, -ζ or –η, are able to induce this cell line to differentiate into mature macrophages when stimulated with phorbol ester.

Immunofluorescence studies with isoform-specific antibodies have been initiated on NIH3T3 and 32D cells which overexpress each PKC isoform. Only a few suitably specific antibodies are currently available, but it is already clear that different isoforms translocate to different subcellular structures when their kinase activity is activated by phorbol esters. This confirms that these similar, but different, serine/threonine kinases have different functions, and these overexpressing lines offer important tools with which to delineate some of these specific roles.

VI. Cyclins:

We are isolating cDNAs for mouse G1/S and G2/M cyclins in order to determine if they can be implicated in tumorigenesis. Two cyclin B1 cDNAs that differ only in the length of their 3' untranslated regions have been isolated from 70Z/3B pre-B cells. As predicted, the expression of cyclin B1 is modulated by physiologically relevant stimuli, i.e., expression increases when cells are stimulated to divide and diminishes when cells are induced to differentiate. Southern hybridization analysis and genetic recombination analysis indicates that cyclin B1 genes or related sequences are located on 9 different mouse chromosomes! Current studies are designed to determine how many of these are effectively expressed. Northern blot studies indicate that G2/M cyclin genes, e.g., cyclin B1 are expressed in all tissues, whereas the mouse homologue to cyclin D1(PRAD) is expressed only in myeloid cells. We are currently searching for a B-cell-specific homologue.

Publications:

Beecham EJ, Mushinski JF, Shacter E, Potter M, Bohr VA. DNA repair in the c-*myc* proto-oncogene locus: possible involvement in susceptibility or resistance to plasmacytoma induction in BALB/c mice. Mol Cell Biol 1991; 11: 3095-3104.

Blank R, Eppig J, Fiedorek FT, Frankel WN, Friedman JM, Jackson I, Mock B. Mouse chromosome 4. Mamm Genome 1991; 1: S51-S58.

Bhatia K, Huppi K, McKeithan T, Siwarski D, Mushinski JF, Magrath I. Mouse bcl-3: cDNA structure, mapping and stage-dependent expression in B lymphocytes. Oncogene 1991; 6: 1569-1573.

Bhatia K, Gutierrez MI, Huppi K, Magrath IT. PCR detection of a neutral CGA/CGG dimorphism in exon 6 of the human p53 gene. Nucl Acids Res 1992; 20: 928.

Clevenger CV, Sillman AL, Hanley-Hyde J Prystowsky. Requirement for prolactin during cell cycle regulated gene expression in cloned T lymphocytes. Mol Endocrinology 1992; in press.

Dlugosz AA, Mischak H, Mushinski JF, Yuspa SH. Transcripts encoding protein kinases C–α, –δ, –ϵ, –ζ and –η are expressed in basal and differentiating mouse keratinocytes in vitro and exhibit quantitative changes in neoplastic cells. Mol Carcinogenesis 1992; in press.

Gutierrez MI, Bhatia K, Siwarski D. Wolff L, Magrath IT, Mushinski JF, Huppi K. Infrequent p53 mutation in mouse tumors with deregulated myc. Cancer Res 1992; 52: 1032-1035.

Hanley-Hyde J, Mushinski JF, Sadofsky M, Huppi K, Krall M, Kozak CA, Mock B. Expression of murine cyclin B1 mRNAs and genetic mapping of related genomic sequences. Genomics; In press.

Kochel T, Mushinski JF, Rice NR. The v-*rel* and c-*rel* proteins exist in high molecular weight complexes in avian and murine cells. Oncogene 1991; 6: 615-626.

Largaespada DA, Kaehler DA, Mischak H, Weissinger EM, Potter M, Mushinski JF, Risser R. A retrovirus that expresses v-abl and c-myc oncogenes rapidly induces plasmacytomas. Oncogene 1992; 7: 811-819.

Mischak H, Kolch W, Goodnight J, Davidson WF, Rapp U, Rose-John S, Mushinski JF. Expression of protein kinase C genes in homopoietic cells is cell-type and B cell-differentiation stage specific. J Immunol 1991; 147: 3981-3987.

Mischak H, Bodenteich A, Kolch W, Goodnight J, Hofer F, Mushinski JF. Mouse protein kinase C-δ: sequence of the cDNA, expression patterns and characterization of the protein. Biochemistry 1991; 30: 7925-7931.

Mock BA, Neumann PE, Eppig JT, Huppi K. Mouse chromosome 15. Mamm Genome 1991; 1: S241-S268.

Mock BA, Neumann PE, Eppig JT, Huppi K. Mouse chromosome 15. Mamm Genome; In press.

Owens JD, Finkelman FD, Mountz JD, Mushinski JF. Nonhomologous recombination at sites within the mouse J_H-C_δ locus accompanies Cμ deletion and switch to immunoglobulin D secretion. Mol Cell Biol 1991; 11: 5660-5670.

Shirakata M, Huppi K, Usuda S, Yoshida K, Sakano H. HMG-1 related DNA binding protein isolated with V-(D)-J recombination signal probes. Mol Cell Biol 1991; 11: 4528-4536.

Weissinger EM, Mischak H, Largaespada DA, Kaehler DA, Mitchell T, Smith-Gill SJ, Risser R, Mushinski JF. Induction of plasmacytomas secreting antigen-specific monoclonal antibodies with a retrovirus expressing v-*abl* and c-*myc*. Proc Natl Acad Sci USA 1991; 88: 8735-8739.

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

PROJECT NUMBER
Z01 CB 08950-10 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular and biological basis of immune recognition

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

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

COOPERATING UNITS (if any) A.B. Hartman, Dept. of Biologics, Walter Reed; D.R. Davies, LMB, NIADKD; J.F. Kirsch, Univ. of Cal. Berkeley; S. Sheriff, T.B. Lavoie, J. Novotny, Squibb Inst. for Med. Res.; G. Glick, Dept. of Chemistry, Univ. Michigan

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, 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

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 prototoype for investigating the specificity of immune recognition. The X-ray structure of HyHEL-5 Fab complexed with a single site-directed mutant of HEL, R68K which reduces the affinity of the complex by a factor of over 10^3, has been solved and refined; these results represent the first time structures have been obtained for 2 antigens, differing at only a single critical residue, complexed to the same antibody, and will provide valuable insight about role of Arginine side chains in protein-protein interactions. We are beginning to define fundamental principles that will allow prediction of function from structure, principles that are critical to such applications as antibody design and vaccine development. We are also approaching the problem of vaccine development by examining structually constrained peptides as possible immunogens for both B-cells and T-cells, and by investigating immunogenicity and protective epitopes in *Shigella flexnerii*. Experiments are in progress to examine the role of circulating antibody-antigen complexes in determinant selection by B-cells and T-cells. Experiments with specific-pathogen-free and conventional BALB/c mice suggest a significant influence of antigenic exposure, particularly viral antigens, on development of the specificity repertoire and plasmacytomagenesis.

Major Findings

A. Molecular Basis of Immunological Regognition in Antibody-Protein Interactions -- Dr. Smith-Gill, Dr. Tuchscherer, Mr. Newman, in collaboration with Dr. Konrad Huppi.

The majority of secondary response monoclonal antibodies (mAbs) specific for hen eggwhite lysozyme (HEL) recognize epitopes that are grouped into larger, nonoverlapping antigenic regions with definable functional boundaries. The apparent antigenic regions show a rough correspondance with the tertiary structure of HEL. In contrast, the majority of early response antibodies (primary and early secondary) fall into one of 3 groups: (*i*) a set localizing to one of the antigenic regions recognized by late response antibodies; (*ii*) a group which recognize at least one region which is distinct from and nonoverlapping with the anitgenic regions recognized by the late antibodies; and (*iii*) a smaller group which all overlap significantly with 2 or more of the late response anigenic regions, *i.e.*, their boundaries intersect the boundaries of secondary response antigenic regions. These data suggest that the topographical arrangements of specificity patterns change during the course of the immune response.

Among the late-response antibodies recognizing the defined antigenic domains, there appear to be contrasting structural stragetics to recognition of a defined antigenic regions. Mr. Newman is studying possible structure-function corelations among a group of antibodies functionally related antibodies to the structurally defined HyHEL-5; it appears that the antibodies represent a variety of structural solutions for a similar epitope. These results contrast with a series of antibodies functionally related to the structurally defined HyHEL-10, all of which appear to use very similar structural solutions to recognition of the same epitope. Ms. Archana Aggarwal is characterizing several additional antibodies in the HyHEL-10 group to test this hypothesis.

Dr. Tuchscherer has been examining in detail the specificity properties of a high-avidity "intersecting" antibody, HyHEL-29. Reactivity patterns with evolutionary and site-directed mutants of lysozyme confirm that it overlap severals antigenic regions, and that its interaction with HEL is very complex. Although antibodies with this type of reactivity pattern are among the highest avidity of any we have isolated to date, they disappear by the midsecondary response. In order to test the hypothesis that changing specificity patterns reflect epitope-directed processing of antigen in B cells as antigen presenting cells, purified antibody/antigen complexes and heteroconjugates made with anti-HEL monoclonal antibodies with different specificities and B cell target antibodies are used as immuogens, and the resulting patterns of antibody and T-cell specificties are examined.

We are continuing detailed analyses of Fab-HEL interfaces utilizing X-ray crystallography (in collaboration with Dr. David Davies, NIAID, and Dr. Steven Sheriff, Bristol-Myers Squibb) and by site-directed mutagenesis of *both* antibody and antigen (in collaboration with Dr. Jack Kirsch, UC Berkeley). Dr. Davies laboratory has successfully solved the X-ray structure of HyHEL-5 complexed with lysozyme containing a single site mutation R68K, which reduces affinity of the complex by a factor of over 10^3. This will be the first structure of a mutated antigen in complex with an antibody generated to the unmutated, native antigen. Analysis of this structure indicates that a molecule of water replaces the "hole" created by reducing the side chain volume by replacing Arg with Lys, and that

this water molecule effectively prevents any salt links between residue 68 and heavy chain Glu residues, which are critical to the affinity of the native complex. Structural studies of HyHEL-5 complexed with the R45K mutant, which reduces affinity by less than 10-fold, are in progress. These studies are yielding important information concerning the role of Arginine side chains in protein-protein interactions.

Recently, experiments in the laboratory of Dr. Alain Paraf (Laboratoire d'Immunologie I.N.R.A., France) have shown an unexpected application for several of our high affinity mAbs to hen egg white lysozyme. These anti-HEL mAbs were effective in detecting chicken lysozyme in heat processed goose or duck "foie gras" to which chicken liver had been added. Further investigations are in progress using these mAbs as a model for a general immunochemical approach utilizing mAbs to identify fraudulent additives in food.

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

Dr. Tuchscherer has also been examining whether constrained, synthetic peptides, synthesized by Dr. Gary Glick (University of Michigan) that resemble short segments within the stucturally determined HyHEL-5 epitope. The peptides have been previously characterized as immunodominant peptides that bind to MHC class II molecules of the H-2^k haplotype. Results to date indicate that constrained peptides can be processed and presented, and recognized by HEL primed T cells. Although to date we have yet to identify a peptide construct which will be recognized by an anti-HEL mAb, we are continuing to explore, in collaboration with Dr. Glick, whether constrained peptides can be recognized by mAbs specific for the native protein. If such a peptide can be identified, it would serve a model for a synthetic peptide vaccine which would stimulate both T cells and B cells.

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. Chaarles 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. Utililizing a novel route of immunization, we have developed serotype-specific murine mAbs recognizing *Shigella* LPS. Experiments are in progress to determine if any of these mAbs will inhibit *in vitro* invasion, and whether they can confer protective immunity when administered passively using a guinea pig model developed by Dr. Hartman for vaccine testing and studying the immune response to this pathogen. We are also beginning a series of mouse-guinea pig fusions, using various antigen delivery modes, including cholera toxin, to produce immune B-cells from various sites in the mucosal immune system. This project is requiring extensive efforts to develop immunoreagents, not currently commercially available, to detect and assay guinea pig mAbs of various heavy chain classes.

C. The Influence of Antigenic Exposure on Immunological Responses -- Dr. Ann McDonald, in collaboration with Dr. Linda Byrd (formerly of LG, currently in Dr. Nash's lab at NIAID/NIH) and Dr. Konrad Huppi.

Dr. Ann McDonald, in collaboration with Dr. Byrd and Dr. Potter, demonstrated that plasmacytoma-susceptible BALB/cAnPt (BALB/c) mice converted to Specific Pathogen-Free (SPF-BALB/c) status have a decreased incidence of pristane-induced plasmacytomas (PCT). Flow cytometry analysis of granuloma cells revealed a general decrease in inflammatory cells at the site of injection and a significant decline in $CD4^+$ lymphocytes, when compared to BALB/c mice housed under more conventional conditions (CON-BALB/c). Dr. McDonald demonstrated a significant increase in $CD4^+$ lymphocytes in the PCT-susceptible BALB/c strain but not in PCT-resistant DBA/2N nor (BALB/c x DBA/2N)F1 mice. These results suggest that plasmacytoma formation is dependent upon exogenous, T-dependent (possibly viral) antigenic stimulation, and that minimal gut flora which was similar in both CON-and SPF-BALB/c mice is not sufficient to render SPF-BALB/c mice susceptible to PCT induction.

Recently, Dr. McDonald has been characterizing the cytokine secretion patterns of peritoneal exudate from PCT resistant and susceptible mice to determine what role T cells may play in the induction of PCT. Pristane-induced levels of interferon-gamma (IFN-γ) greater than controls were found in the peritoneal lavages of BALB/c mice at all time points tested after pristane injection, but not in DBA/2N or indomethacin-treated BALB/c mice. In contrast, pristane injection increased levels of interleukin-5 (IL-5) in DBA but not BALB/c mice. SPF-BALB/c mice showed no detectable secretion of IFN-γ, IL-4 or IL-5 (manuscript submitted for publication). We propose that T cells contribute to the development of PCT by promoting the development of plasma cells within oil granulomatous tissues through cytokine secretion of IFN-γ and chronic macrophage activation. Future work requires *in vivo* depletion of $CD4^+$ cells and/or IFN-γ to determine whether T cells are an absolute requirement for plasmacytomagenesis and how they contribute to tumor development. Additional studies performed in collaboration with Drs Huppi and Byrd are looking at TCR variable region gene expression of oil granuloma T cells. Limited expression of TCR genes or a lack of expression could provide clues as to the T cell stimulus in oil granulomatous tissues.

SPF-BALB/c mice are widely used in immunological research and yet the above results suggest an altered capacity to respond to an immunologic challenge as a result of prolonged antigenic deprivation. Recent studies are focusing on the ability of SPF-BALB/c mice to respond to the soluble antigen, hen egg lysozyme (HEL). When injected in complete Freund's adjuvant subcutaneously at a conecntration of 250ug/mouse, there was no difference in the ability of draining lymph nodes from either SPF- or CON-BALB/c mice to proliferate in response to antigen, *in vitro*. However, only CON-BALB/c mice produced detectable levels of IFN-g after HEL stimulation. Interestingly, SPF-BALB/c lymph node cells exhibited greater levels of proliferation and IFN-g secretion to Con-A stimulation than CON-BALB/c lymph node cells. Moreover, SPF-BALB/c mice required greater concentrations of HEL before proliferative responses were detected. Future studies, will continue to define differences in immune responsiveness between SPF- and CON-BALB/c mice. In addition, studies will begin on the reconstitution of the immune response in SPF-BALB/c mice by exposure to defined antigens and their subsequent response to immunologic challenge or PCT development.

Founder pairs of the SPF-BALB/cPt colony which were derived by Dr. Byrd from the Hazleton CON Balb/cPt have been successfuly introduced to the FCRF SPF facility, and are being used to establish pedigree lines. These lines will be used for breeding mice for future experiments requiring SPF BALB/cPt mice, freeing facilities at the Hazleton colony currently being used for production of SPF mice.

Publications

Byrd LG, McDonald AH, Gold LG, Potter M. Specific pathogen-free BALBc/AN mice are refractory to plasmacytoma induction by pristane. J Immunol 1991;147:3632-37.

Smith-Gill SJ. Protein-protein interactions: Structural motifs and molecular recognition. Curr Op Biotech 1991;2:568-75.

Weissinger EM, Mischak H, Largaespada D, Kaehler DA, Mitchell T, Smith-Gill SJ, Risser R, Mushinski JF. Induction of antigen-specific plasmacytomas with an ABL-MYC retrovirus. Proc Natl Acad Sci 1991;88:8735-39.

Lavoie TB, Drohan WN, Smith-Gill SJ. Experimental analysis by site directed mutagenesis of somatic mutation effects on affinity and fine specificity in antibodies specific for lysozyme. J Immunol 1992;148:503-13.

Kam-Morgan LNW, Kirsch JF, Lavoie TB, and Smith-Gill SJ. Site idrected mutagenesis as a tool in the analysis of protein-protein interactions. Meth Enzymol 1992; in press.

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

Newman MA, Mainhart CR, Mallett CP, Lavoie TB, Smith-Gill SJ. Patterns of antibody specificity during the BALB/c immune response to hen egg white lysozyme. J Immunol 1991; in press.

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

PROJECT NUMBER
Z01 CB-08952-06 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Retrovirus-induced acute myeloid leukemia in mice

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

P.I.:	L. Wolff	Senior Investigator	LG, NCI
	R. Mukhopadhyaya	Visiting Fellow	LG, NCI
	K. Nason-Burchenal	Microbiologist	LG, NCI
	R. Koller	Biologist	LG, NCI

COOPERATING UNITS (if any)
J.F. Mushinski, LG, NCI; M. Sitbon, Hopital Cochin, Institute National de la Sante et de la Recherche Medicale U152 Centre National de la Recherche Scientifique, UA628, Paris, France

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.8	3.8	0.0

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 has developed a murine model for acute myeloid leukemia and the primary objective has been to understand the mechanisms involved in the development of this disease. Since the leukemia is induced by a combination of retroviruses and pristane-induced inflammation, the major focus has been to determine the role of each of these contributing factors on oncogene activation, and on cellular transformation, expansion and trafficking. Our recent studies on c-*myb* activation have determined that the protooncogene can be activated by two viral-induced mechanisms in promonocytic leukemias which are different from those previously found. These were activations found in FB29-induced promonocytic leukemias having two new sites of integration. Of paricular interest was the fact that the aberrant Myb protein expressed in one of these tumors has the smallest N-terminal truncation thus far determined for a leukemia-associated Myb. Another study aimed at understanding the temporal relationship of *myb* activation (using RT-PCR technology) to the development of disease determined that the *myb* protooncogene is activated very early in the disease process. Cells with this activation are most prevalent in the bone marrow at three weeks post-virus infection and disseminate during disease progression to other sites including the spleen, liver, and bone marrow. By 8 weeks, 100% of mice harbor cells that have undergone insertional mutagenesis, although it has been shown previously in numerous experiments that only approximately 50% of mice succumb to disease. Progress has also been made in the determination of viral genes specifically required for leukemia development. Analysis of recombinant viruses indicate specific roles for *gag* and *env* genes in development of promonocytic leukemia in BALB/c mice.

Major Findings:

New sites of virus integration in murine promonocytic leukemias: evidence for alternate mode of activation of c-*myb* by insertional mutagenesis. Insertional mutagenesis of c-*myb* occurs in promonocytic leukemias induced by Moloney MuLV (MML) and Ampho 4070A (AMPHO-ML). Recently, it was found that under similar conditions FB29 can induce promonocytic leukemias as well. In some of these leukemias, virus has integrated at sites not previously observed in MML or AMPHO-ML. These sites were either at the 5′ end of c-*myb* upstream of exon 2 (in the first intron) or in exon 9, towards the 3′ end of the gene. When the structure of the RNAs, expressed in leukemias induced by FB29, was studied by sequencing partial cDNA products generated by RT-PCR amplification, it was found that tumors with integrations in the first intron express virus-*myb* fusion RNAs. In these RNAs *gag* has been joined to the beginning of *myb* exon 2 and this occurs through the use of a cryptic splice donor in *gag*. Since we were interested in characterizing the Myb protein structure in these leukemias, which have intron 1 integrations and which express fusion RNAs with gag/exon 2 junctions, we carried out in vitro transcription and translation using a cDNA template in which we generated mutations at potential translation start sites. In addition, we examined the aberrant Myb expressed in relevant leukemic cell lines. Our results demonstrate that at least one of these leukemias (R1-4-10) with a unique integration expresses Myb with the smallest N-terminal truncation observed so far in promonocytic leukemias; translation begins at an ATG within c-*myb* exon 2, leading to loss of only 20 amino acids from the N-terminus. Unlike the proteins with larger truncations of 47 and 71 amino acids in MML, this protein has an intact DNA binding region. In addition, unlike previously described tumor-specific Mybs this protein does not contain N-terminal amino acids encoded by *gag*. However, this protein shares with all N-terminally truncated Mybs so far studied, a loss of a casein kinase II (CKII) phosphorylation site believed to be involved in regulation of DNA binding. Although, leukemia R1-4-10 expresses RNA sequence with the potential to encode the CKII phosphorylation site, it does not appear to be translated as a part of the final Myb. Our data further emphasize the importance of the loss of this site to formation of a transforming Myb.

RT-PCR approach for monitoring the formation and trafficking of preleukemia cells with activated c-*myb*. Insertional mutagenesis of c-*myb* by Moloney MuLV occurs in 100% of promonocytic leukemias (MML) induced by injection of virus and pristane. We have found that two leukemia specific *gag-myb* mRNAs in MML provide molecular markers for early detection of preleukemic cells in vivo. Previously, we reported preliminary development of an RT-PCR technique for detecting cells in vivo which have undergone retroviral insertional mutagenesis of c-*myb*. The technique involves reverse transcription and amplification of specific *gag-myb* mRNA present in these cells. This past year this technique was highly refined to detect one cell with activated c-*myb* in a population of 10^7 cells. This technique involves two rounds of amplification combined with hybridization using oligonucleotides specific for the *gag-myb* junctions. It was necessary to take several precautionary steps to prevent false positives. We feel that the technique is more sensitive and specific than any we have seen reported in the literature. After refining the technique, a large experiment was set up to determine the tissue location of cells with activated c-*myb* that are a consequence of pristane treatment and

Moloney MuLV inoculation i.v. Cells with *gag-myb* mRNA were detected as early as 2 wks post virus-inoculation and could be found in greater than 80% of the mice by 3 weeks and 100% of mice by 8 weeks in spite of the fact that acute leukemia is not evident until 3 or 4 months post virus-inoculation. Early detection in the bone marrow of cells with aberrant c-*myb* RNA suggest that this hematopoietic organ is the site for initial development of MML. The data also indicate that at an intermediate stage, these cells disseminate from the bone marrow to the spleen, liver and peritoneal cavity. A particularly intriquing finding was that all mice which receive virus inoculations have preleukemic cells, even though only 45-55% of mice succumb to disease at 3-4 months post-virus infection.

Analysis of recombinant viruses indicate specific roles for *gag* and *env* genes in development of promonocytic leukemias. Moloney murine leukemia virus (MuLV) is capable of inducing promonocytic leukemias in 50% of adult BALB/c mice that have received peritoneal injections of pristane, but Friend MuLV strain 57, is nonleukemogenic under similar conditions. It was shown earlier that these differences could not be mapped to the U3 region of LTR, indicating the probable influence of structural genes or R-U5 sequences. In the present study, reciprocal recombinant viruses containing exchanged structural genes and R-U5 sequences from these two closely related viruses were studied for differences in ability to induce disease. Adult BALB/c mice, inoculated with the recombinant retroviruses and pristane, were monitored for disease and for replication of virus in hematopoietic tissue. DNAs from tumors were subsequently analyzed for rearrangements of c-*myb*, a characteristic feature of these promonocytic leukemias. Results showed that constructs of Friend MuLV with substitutions of R-U5/*gag* or *env* from Moloney MuLV were leukemogenic with incidences of 13% and 4%, whereas a construct with a substitution from Moloney *pol* was still nonleukemogenic. The importance of both of these genetic regions in promonocytic leukemias induced by Moloney MuLV could be further demonstrated by analyzing the results of virus, FM5GE, which contained both R-U5/*gag* and *env* genes from Moloney MuLV and which gave an incidence of 62%. Recombinants in which individual Friend virus genes were evaluated for their ability to attenuate Moloney MuLV disease also supported the fact that R-U5/*gag* and *env* genes both account for differences the in leukemogenicity of these two viruses.

Publications:

Wolff L, Koller R, Davidson W. Acute myeloid leukemia induction by Amphotropic retrovirus 4070A: Clonal integration within the c-myb in some but not all leukemias. J Virol 1991;65:3607-16.

Gutierrez MI, Bhatia K, Siwarski D, Wolff L, Magrath IT, Mushinski JF, Huppi K. Infrequent p53 mutation in mouse tumors with deregulated myc. Cancer Res 1992:52:1032-5.

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

PROJECT NUMBER
Z01 CB 08953-02 LG

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Effects of individual genes on hematopoietic cell differentiation and function

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

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

COOPERATING UNITS (if any)
Dr. C.C. Chan, Dr. F. Roberge, NEI, NIH; Dr. M. Sneller, LCI, NIAID, NIH; Dr. J. Allison, Univ. of California, Cancer Research Lab., Berkeley, CA

LAB/BRANCH
Laboratory of Genetics

SECTION
Molecular Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	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.)

Mice homozygous for lpr and gld develop profound lymphadenopathy characterized by the accumulation of two functionally impaired T cell subsets, a predominant $B220^+$ $CD4^-CD8^-$(DN) population and a minor $CD4^+B220^+$ subset. Previous studies of diseased lpr and gld mice revealed that enlarged LN also contain elevated numbers of conventional T and B cells and an increased proportion of memory-like $CD4^+$ $B220^-$ T cells. In the past year we have continued to explore the effects of lpr and gld on T cell selection, growth and activation. These studies revealed that primed $CD4^+$ and $CD8^+$ T cells are detected in increased numbers in lpr LN by 4 wk of age and accumulate progressively thereafter. Two types of memory cells distinguished by differences in ontogeny, phenotype and the spectrum of cytokines secreted post-stimulation were identified. Examination of TcR V_β gene utilization revealed no skewing of the V_β repertoire that would implicate autoantigens as a primary stimulus for the activation of $CD4^+$ or $CD8^+$ T cells early or late in disease. The pattern of expression of CD69 on $B220^+$ DN T cells provided further evidence that these cells may be chronically stimulated in vivo but unable to progress through the cell cycle. The functional anergy of DN T cells was reversed by cross-linking TcR α/β and CD28 in the presence of PMA, indicating that the blockage in signal transduction via the TcR complex can be overcome in the presence of a strong costimulatory signal. One unifying hypothesis to explain lymphoproliferative disease is that the lpr and gld mutations result in defective regulation of the size of the pool of primed T and B cells. DN T cells may arise from chronically stimulated, abnormally long-lived $CD4^+$ and $CD8^+$ T cells that down-regulate the expression of CD4, CD8 and TcR α/β, switch to the B220 isoform of CD45, and become resistant to activation. Selective accumulation of DN T cells may result from a combination of events including chronic low level stimulation, increased lifespan and the ready availability of growth factors. Experiments to test these predictions are in progress and are outlined.

Major Findings:

T cell abnormalities associated with the expression of lpr and gld

C3H mice homozygous for lpr and gld develop strikingly similar diseases characterized by profound lymphadenopathy, autoantibody production and premature death. Lymphadenopathy results predominantly from the accumulation of a unique population of functionally anergic $B220^+CD4^-CD8^-$ ($B220^+$ 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 impaired CD4 $dull^+$ $B220^+$ T cells. Our long term goals are: 1) to determine the origin of $B220^+$ T cells, 2) to elucidate the mechanisms leading to the functional impairment of $B220^+$ T cells, 3) to further characterize the lymphocytes that accumulate and to understand the mechanisms leading to their accumulation, and 4) to determine how the various lymphocyte subsets contribute to the development of autoimmune disease. The approaches we have taken over the past year to address these issues are outlined below.

Evidence for early, polyclonal activation of lpr and gld T cell subsets

Recently, we reported that a high proportion of $CD4^+B220^-$ T cells in diseased lpr and gld mice have the hallmarks of primed or memory T cells. In the past year we further investigated the extent, ontogeny and possible causes of T cell activation. The criteria used to identify primed or memory T cells included activation-dependent increases in the expression of CD44, LFA-1 and the early activation antigen, CD69, and decreases in the expression of MEL-14 and CD45RB, as well as quantitative differences in the in vitro production of IFN-γ and TNF-α by stimulated cells. A comparison of TCR V_β gene utilization by lpr T cell subsets also was undertaken to determine if T cell activation was driven by inappropriate responses to self antigens. The results showed that T cell activation in lpr and gld mice was widespread and complex. $CD8^+$ T cells from diseased mice exhibited a similar pattern of activation to $CD4^+B220^-$ T cells. The activation of these two subsets occurred in parallel, was in evidence by 4 to 6 wk of age, and was both chronic and progressive. The proportions of $CD44^{hi}LFA^{hi}$ $CD4^+B220^-$ and $CD8^+$ T cells increased steadily between 4 and 20 wk of age, but changes in T cell growth, Mel-14 and CD45RB expression and cytokine secretion were not observed until the mice were older than 11 wk. A very different pattern of activation was observed for $B220^+$DN T cells and $CD4^+B220^+$ T cells. At all ages, these cells were $CD44^{hi}$Mel-14^{hi} and 60-75% were $CD69^+$. The appearance of CD69 appeared to be stimulus-dependent rather than constitutive, suggesting that these cells, too, may be chronically stimulated in vivo. In keeping with their anergic state, DN T cells responded poorly to cross-linking of CD69. The stimuli inducing chronic activation of $CD4^+B220^-$ and $CD8^+$ T cells are unlikely to include inappropriate reactions to autoantigens since there was no evidence for selective accumulation of $CD4^+$ or $CD8^+$ T cells bearing particular V_β genes or Mls^c-reactive $V_\beta3^+$ or IE-reactive $V_\beta11^+$ T cells that normally are deleted in the thymus or anergized in the periphery of C3H mice. By comparison, there was some skewing of the V_β repertoire of DN T cells evidenced by a consistent decrease in the proportions of $V_\beta6^+$ cells and an increase in the proportions of $V_\beta9^+$ T cells. We have yet to determine whether the increase in $V_\beta9^+$ T cells is indicative of a response

to self-antigens other than Mls^c or IE. Comparisons of the total percentages of cells reacting with mAb for 9 different V_β antigens revealed a close similarity between $CD4^+$ and DN T cells. One interpretation of this data is that $B220^+$ DN T cells are derived from primed $CD4^+B220^-$ T cells, possibly via a $CD4^+B220^+$ intermediate.

Developmental relationships among T cell subsets

To examine the developmental relationship between $CD4^+$ and DN T cells more directly, we reconstituted immunodeficient C.B-17-scid mice with unfractionated BM, LN or spleen cells from young BALB-gld without disease or purified $CD4^+$ $B220^-$, $CD4^+B220^+$ or $B220^+$ DN T cells from mice with advanced lymphoproliferative disease. Preliminary results confirmed that BM cells will transfer the disease, and also demonstrated that unfractionated LN cells from young BALB-gld mice are sufficient to transfer disease. Thus, mice reconstituted with gld LN cells developed lymphoproliferative disease similar to that of BALB-gld mice. The LN of these mice contained a predominant population of $B220^+$ DN T cells as well as smaller numbers of $CD4^+B220^-$, $CD4^+B220^+$ and $CD8^+$ T cells and B cells. By comparison, DN T cells failed to transfer disease and did not give rise to significant numbers of $CD4^+$ or $CD8^+$ T cells or B cells.

Very recently it was reported that the wild-type allele of lpr is equivalent to fas, a gene that encodes a cell surface receptor that, following engagement, will induce cell death by apoptosis. In lpr mice, there is a rearrangement in the second intron of fas that prevents or greatly decreases transcription of the gene. Other investigators demonstrated that only previously activated lymphocytes are susceptible to Fas-mediated apoptosis. This observation led us to predict that in normal mice, Fas may play a role in regulating the size of the pool of primed T and B lymphocytes and that the absence of Fas expression on activated lpr T and B lymphocytes may provide an explanation for the progressive accumulation of these cells in lpr mice. We have generated molecular probes for murine and human fas and presently are using these to examine fas mRNA expression in lpr and gld lymphocyte populations. In collaboration with Dr. Michael Sneller we also are examining fas mRNA expression in the lymphocytes of two pediatric patients in the Clinical Center who have a lymphoproliferative disease with many similarities to that of lpr and gld mice.

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

$B220^+$ DN T cells are an enigmatic population that fails to respond to a wide variety of stimuli including crosslinking of the TcR complex, but has many of the hallmarks of previously activated T cells, such as high levels of expression of CD44, LFA-1 and CD69 and constitutive phosphorylation of the zeta chain of the TcR complex. One possible explanation for the functional anergy of DN T cells is that they lack the costimulatory signals required to mount a productive immune response. Previously, we reported the lack of expression of one known costimulatory molecule, CD2, on DN T cells. Recently, in collaboration with Dr. Jim Allison, we examined DN T cells for impaired expression or function of another costimulatory molecule, CD28. Studies of mRNA expression and surface antigen expression indicated that $B220^+$ DN T cells expressed higher than normal levels of CD28. In costimulation assays, highly purified $B220^+$

TcR α/β^+ DN T cells cultured with mAb to CD28 in combination with TcR crosslinking or PMA and ionophore, both proliferated and secreted IFN-γ and IL-2 but not IL-4 or IL-10. Quantitative and kinetic differences in the proliferative responses and cytokine production by normal and $B220^+$ DN T cells in the costimulation assay suggested that $B220^+$ DN T cells either respond less efficiently than normal T cells or that only a subpopulation of DN T cells is responsive.

The ligand for CD28, known as B7, is expressed on antigen-presenting cells. Using PCR analysis we demonstrated that B7 mRNA is present in higher than normal levels in lpr and gld LN cells. The expression of B7 and functional CD28 on lpr and gld LN cells suggests that conditions may be favorable for DN T cells to respond to antigenic stimulation in vivo. Preliminary experiments indicating that $V_\beta 8^+$ and $V_\beta 6^+$ DN T cells respond to stimulation with staphylococcal enterotoxin B in vivo provide some support for this prediction. Thus, $B220^+$ DN T cells may accumulate as a result of the continuous conversion of primed T cells to DN T cells and a low level of antigen-driven proliferation.

The role of cytokines in lymphoproliferative disease

In a published report we demonstrated that the primed $CD4^+$ and $CD8^+$ T cells that progressively accumulate in the lymphoid organs of lpr and gld mice have the potential to secrete high levels of IL-10, IFN-γ and TNF-α following stimulation. These cytokines have pleiotropic effects on hematopoietic cells and conceivably could have important secondary influences on the growth or survival of conventional T and B lymphocytes and $B220^+$ DN T cells. To test this hypothesis, C3H-gld mice presently are being treated continuously with encapsulated hybridoma cells producing antibodies specific for IFN-γ and TNF-α. Similar experiments are planned with mAb to IL-10.

Evidence for autoimmune eye disease in BALB-gld mice

A high proportion of male and female BALB-gld mice older than 5 mon develop severe inflammatory keratitis of the eye. This condition, which has not been observed in C3H-gld mice or C57Bl/6-, C3H- or MRL-lpr mice, closely resembles Mooren's corneal degeneration, a rare autoimmune disease of the eye in humans. A detailed, collaborative study of this disease is being undertaken with Drs. Chan and Roberge of the Eye Institute.

Our future research will continue to focus on the developmental relationships among lpr and gld T cell subsets and the mechanisms promoting the accumulation of primed T and B lymphocytes and relatively anergic $B220^+$ DN T cells.

Publications:

Davidson WF, Calkins C, Hugin A, Giese T, Holmes KL. Cytokine secretion by C3H-lpr and -gld T cells. Hypersecretion of IFN-gamma and TNF-alpha by stimulated $CD4^+$ T cells. J Immunol 1991;146:4138-48.

Wolff L, Koller R, Davidson WF. Acute myeloid leukemia induction by Amphotropic retrovirus 4070A: clonal integration within the c-myb in some but not all leukemias. J Virol 1991;65:3607-16.

Makino, M, Davidson WF, Fredrickson TN, Hartley J, Morse HC III. Effects of non-MHC loci on resistance to retrovirus-induced immunodeficiency in mice. Immunogenetics 1991;33:345-51.

Davidson WF, Pierce JH, Holmes KL. Evidence for a developmental relationship between $CD5^+$ B cells and macrophages. Ann NY Acad Sci 1992;651:112-29.

Mischak H, Kolch W, Goodnight J, Davidson WF, Rapp U, Rose-John S, Mushinski JF. Expression of protein kinase C genes in hemopoietic cells is cell-type and B cell differentiation stage specific. J Immunol 1991;147:3981-87.

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

Giese T, Davidson W. Evidence for early onset and polyclonal activation of $CD4^+$ and $CD8^+$ T cells in mice homozygous for lpr and gld. J Immunol 1992; in press.

SUMMARY STATEMENT

LABORATORY OF BIOCHEMISTRY

DCBDC, NCI

OCTOBER 1, 1991 TO SEPTEMBER 30, 1992

INTRODUCTION

The Laboratory of Biochemistry has lived up to its tradition of attracting and supporting a number of young outstanding independent investigators. These scientists conduct diverse research projects which span a broad array of disciplines encompassing molecular and developmental biology, genetics, and biochemistry. The projects reflect the different interests of the fifteen groups which constitute the laboratory. Two members of the laboratory were recognized for their outstanding contributions. Dr. Carl Wu received the Young Investigator of the Year Award (1992) from American Society for Biochemistry and Molecular Biology and Dr. Claude Klee received the WISE Lifetime Achievement Award, the Meritorious Award, and was elected to the Institute of Medicine, National Academy of Sciences.

During the last year, emphasis has been on the creation of a new Drosophila genetics facility since many members of the laboratory will use genetic techniques in Drosophila to study the function of proteins in vivo. At the same time we took advantage of the proximity of a first class structural biology laboratory in the Chemical Biophysics Laboratory (NIADDK) to initiate collaborative projects to study protein structures. Common approaches to different problems have fostered fruitful interchanges between the members of the laboratory and other scientists at NIH. There is not a day without a seminar or group meeting despite the fact that space limitations make it often difficult to find a free seminar room.

Dr. Samuel Wilson left the laboratory in December 1991 to become Director of the Sealy and Smith Center for Molecular Sciences and Professor of Biochemistry at the University of Texas in Galveston. We were sorry to lose one of the most productive members of the laboratory but were fortunate to attract a young investigator with superb training in Drosophila genetics, Dr. Mark Mortin, to fill the position vacated by his departure and thereby extend the scope of our research interests and broaden our expertise.

Mark Mortin joined the laboratory in October 1991 and established an independent research program centered on the study of RNA polymerase II in Drosophila. With the help of Carl Wu, he has started to set up a Drosophila genetics facility. This unit is already being used by five groups in the laboratory. We are all very grateful to Mark who generously shares his time and expertise with us. We anticipate that this facility will be a great asset for the laboratory. It has already attracted three outstanding young postdoctoral fellows interested in pursuing a career in Drosophila genetics.

We were particularly fortunate this year to have been allocated a large equipment budget which enabled us to purchase a number of state-of-the-art pieces of equipment such as an ultracentrifuge, a mass spectrometer, a

phosphorimager, and to update our computer facilities. Creation of the Drosophila genetics facility would not have been possible without the generous support of our Division Director.

REGULATION OF GENE EXPRESSION

Regulation of gene expression remains a major focus of interest with six independent groups whose research projects are devoted to different aspects of this subject.

A basic approach to this problem is being taken by Mark Mortin. Regulation of RNA polymerase II, which is required for the synthesis of all messenger RNAs, is a prerequisite for regulation of gene expression. Both the structure of this multimeric enzyme and its mechanism of action are poorly understood. Mark Mortin is using a genetic approach to identify subunits of and proteins that interact with Drosophila RNA polymerase II. Using mutations that he has generated in the two largest subunits, he has identified second-site mutations in genes that encode proteins likely to interact with RNA polymerase II. Forty-one mutations that either enhance or suppress mutant phenotypes elicited by the initial RNA polymerase II mutations have been isolated. One major focus of his laboratory is to clone two of the genes that are believed not to encode subunits of RNA polymerase II. In addition, seven of the 41 second-site mutations map in the second-largest subunit and suppress lethality caused by a specific mutation in the largest subunit. During the next year efforts will be made to sequence these putative point mutations to determine whether they identify contact points between the two largest subunits.

Carl Wu and his colleagues have continued to make original contributions to the study of three sequence-specific DNA-binding proteins in Drosophila, the heat shock transcription factor, HSF, and two factors which regulate the segmentation gene fushi tarazu, FTZ-F1 and tramtrack. Following the cloning of the HSF gene from humans in the previous year, they undertook a comparative study of HSF function in humans and Drosophila. Considerable progress has been made in the molecular genetic analysis of the regions of the HSF protein that are important for its regulation. The most interesting result regarding HSF regulation has been the demonstration of the importance of the C-terminal leucine zipper motif in maintaining the inactive HSF conformation. Another significant achievement was the successful demonstration of the proposed function of the tramtrack protein as a repressor of the ftz gene. An additional highlight came from the immunostaining analysis of FTZ-F1 on Drosophila polytene chromosomes which revealed a new function for FTZ-F1 as an intermediate regulator of genes expressed at the onset of metamorphosis. These findings further affirm the phenomenon of regulatory pleiotropy, and suggest that many, if not the majority of transcription factors regulating developmental genes are utilized repeatedly throughout the life of the organism to serve different regulatory pathways.

The regulation and mode of action of the MyoD family of myogenic regulatory factors in the vertebrates and in Drosophila continues to be the focus of interest of Dr. Paterson and his colleagues. They have isolated four of the avian myogenic factor genes which are homologous to mouse MyoD, myogenin, myf5, and MRF4. During his sabbatical leave in Dr. Walter Gehring's laboratory in Basel, Switzerland, Dr. Paterson isolated and characterized the single Drosophila homologue, Dmyd, in order to take a genetic approach to study myogenesis. Each of the factors encoded by members of this gene family, from C.

elegans to man, shares a structural motif containing a basic DNA binding region joined to a helix-loop-helix (HLH) dimerization domain which is essential for function. The avian and mouse factors, when transfected into mouse 10T1/2 fibroblasts, convert these cells to muscle, activate cotransfected muscle-specific promoter CAT constructs, and dimerize with the E-family of HLH proteins to bind the E-box consensus sequence, CANNTG. Studies on the Drosophila factor, Dmyd, which despite its strong homology to mouse MyoD does not convert 10T1/2 cells to muscle, has helped to define particular hydrophilic residues in the HLH domain that are essential for efficient dimerization of MyoD and the E-protein, E12, which is apparently required to promote muscle differentiation. These observations led Dr. Paterson and his colleagues to propose a parallel four-helix bundle model for the general structure of the heterodimer. These studies were facilitated by the development of a novel baculovirus expression system yielding sufficient amounts of protein, and enabled them to study the role of protein phosphorylation in the functional regulation of these myogenic factors.

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

Dr. Hamer's group has completed a ten-year study of gene regulation by metal ions with the discovery of a new nuclear regulatory factor, UPC31, that activates the reduction and uptake of Cu ions by yeast cells. Interestingly, the amino-terminal domain of UPC31 is homologous to the Cu- and DNA-binding domain of ACE1, the Cu-dependent activator of metallothionein (CUP1) gene transcription in yeast. Thus the basic pathway for Cu homeostasis in yeast cells is now known: Cu → UPC31 → uptale & utilization → ACE1 → CUP1 → sequestration. A remarkable feature of this pathway is that the three critical proteins - UPC31, ACE1 and CUP1 - all share a common structural feature, the "copper-fist".

More recently, Dr. Hamer has shifted his attention to a new research problem, the sexual determination of brain structure and function. Genes that influence sexual development in man are being sought by genetic segregation, association and linkage studies of sexual orientation. These studies have been combined with an intramural, multi-institute medical research project on "Genetic Factors and Interrelationships for Sexual Orientation, Susceptibility to HIV and Kaposi's Sarcoma, Alcoholism and Related Psychopathology, and Histocompatibility Antigens," for which Dr. Hamer is the principal investigator. Attempts to detect and isolate genes that contribute to courtship behavior and sexual dimorphism in Drosophila and rats are also under way.

The role of DNA methylation in the modulation of gene expression is a major focus of interest in Dr. Kuff's laboratory. As an experimental system, his group is using a type of envelope-deficient mouse retrovirus, the intracisternal A-particle (IAP). The promoter activity of IAP long terminal repeats (LTRs) is known to be inhibited both in vivo and in cell-free systems by methylation of

specific CpG sites located in sequence motifs that are binding sites for nuclear factors. One of these factors, a heterodimeric protein designated EBP-80, was shown by Dr. Kuff's group to be indistinguishable from a previously isolated protein, Ku, identified by antibodies in the sera of certain autoimmune patients. Ku has been known to bind tightly to the ends of duplex DNA. Dr. Kuff's group has now shown that EBP-80/Ku can also recognize single to double strand transitions in DNA, such as palindromic extrusions and internal bubbles of non-homology. Binding of this protein could be sensitive to conformational alterations in DNA induced by methylation at certain sites. A related question is the basis for selectivity of IAP gene activation in vivo. Recently, this laboratory has shown that a small and sequence-specific subset of IAP elements accounts for the great majority of IAP-related transcripts in normal lymphocytes of certain inbred mouse strains. The genomic distribution of these elements in various inbred strains and the basis for their highly selective activation are under study.

PROTEINS AND THE CONTROL OF CELLULAR PROCESSES

Different approaches to the regulation of cell function are taken by five other groups who study specific proteins involved in different cellular processes. The overlap between these research projects and those described above is becoming evident since many of the proteins under study have now been shown to be involved in the regulation of gene expression.

Dr. Shelby Berger and her colleagues are making steady progress in understanding prothymosin α gene expression and the essential role of the protein in cell division. The protein is encoded in a gene family which consists of one functional gene and five processed pseudogenes. Although the latter possess consensus regulatory signals for transcription and translation and, in some cases, highly conserved open reading frames, none is expressed. The functional gene is alternatively spliced to yield two transcripts, the most common of which violates consensus rules for selection of the splice acceptor site. The remaining 10% of the transcripts, which are found in the eight tissues examined and in normal and transformed cells, contain an insertion consisting of one glutamic acid residue. As a result, the Berger laboratory has refined the rules for splice acceptor selection. They believe that a G residue immediately upstream of an AG dinucleotide abrogates splicing except when two GAG motifs occur in tandem. The site at which the protein(s) are posttranslationally modified has been identified. Major efforts are now devoted to identifing the function of prothymosin α and the putative role of c-myc in the selective transcriptional activation of prothymosin α.

The long term commitment of Dr. Klee's group to the study of the activation of the calmodulin-regulated protein phosphatase, calcineurin, by calmodulin was rewarded last year by the report of Dr. Schreiber and his colleagues at Harvard that the inhibition of calcineurin's phosphatase activity by immunosuppressants is responsible for their inhibition of T cell activation. Collaborative efforts of Dr. Klee with the laboratories of Drs. Schreiber and Burakoff have shown that immunosuppressants inhibit calcineurin in vivo and that inhibition of calcineurin activity parallels their inhibition of Il-2 expression. The immunosuppressant binding site has been localized to the carboxyl terminal two thirds of calcineurin A which also harbors the catalytic center and the calcineurin B-binding site. Another important development has resulted from a collaborative project with Drs. Bax and Ikura at NIH. This is the elucidation

of the structure of the complex of calmodulin with a calmodulin-binding peptide. These studies helped to define structural requirements for calmodulin target protein interactions and revealed the importance of the flexibility of the central helix in this interaction.

The research of Dr. Wagner and colleagues concerns the regulation of secretion by Ca^{2+} and GTP-binding proteins. They have made the novel observation that modification of chromaffin cells with pertussis toxin results in a large decrease in cortical actin filaments. This depolymerization of F-actin may account for the increased secretory activity of pertussis toxin-modified chromaffin cells. This decrease in F-actin, apparently independent of changes in known second messengers, may result from a direct interaction of a G-protein with the cytoskeleton. They have also isolated a cytosolic protein, homologous to brain 14-3-3 protein, which appears to play an important role in the secretory response.

Dr. Peterkofsky's laboratory has identified IGFBP-1 and IGFBP-2 as the circulating insulin-like growth factor binding proteins that are induced in vitamin C deficient and fasted guinea pigs. The temporal pattern of induction of the mRNAs for these proteins in liver, compared to the decline in type I collagen mRNA levels in bone and skin, is compatible with the proposed role of the IGFBPs as inhibitors of collagen synthesis during the nutritional deficiencies.

Pursuing a project initiated during a sabbatical year in Dr. Margulies's laboratory, Dr. Mage has concentrated his efforts on the characterization of two recombinant single chain Class I MHC molecules, which have their ß2-microglobulin (ß2m) domain covalently linked to the heavy chain. One recombinant molecule has a wild type amino acid sequences. The other is a mutant molecule which lacks three salt bridges in the antigenic peptide-binding domains. Both molecules bind endogenous peptides, indicating that they are correctly folded, and suggesting that a ß2m-free heavy chain/peptide complex is not an obligate intermediate in assembly. When pulsed with an antigenic peptide from the HIV gp120 envelope protein, both molecules can present it to an H-$2D^d$ restricted antigen-specific T hybridoma. Thus, the recombinant molecules are biologically active. Because the single chain molecule, despite a tethered ß2m, retains sufficient flexibility to be loaded <u>in vitro</u> with antigenic peptides, it may be useful for structural studies, and as an intermediate in the formation of toxin-conjugates and T cell vaccines.

ORGANIZATION OF THE HUMAN GENOME

Drs. McBride and Singer continue to devote their efforts to the study of the organization of the human genome.

Dr. McBride's research projects fall within three categories. The first involves the chromosomal localization of cloned genes, tumor breakpoints, and other cloned sequences by Southern analysis of human-rodent somatic cell hybrids, in situ hybridization, and genetic linkage analysis in 40 large CEPH families. Twenty additional cloned genes have been mapped during the past year. The second involves the use of linkage analysis for mapping and identifying specific tumor susceptibility, and other disease genes in families segregating these genes. Among many others, it includes the mapping of the neurofibromatosis 2 (NF2) gene in collaboration with Drs. D. Parry and R.

Eldridge, and the mapping of disease genes in several genetic skin diseases in collaboration with Drs. P. Steinert and S. Bale. A third novel experimental approach was designed to contruct high resolution genetic and physical chromosome maps. The method is based on the use of trinucleotide repeats to isolate, clone, and sequence chromosome regions flanking microsatellite DNAs. By PCR amplication of these microsatellite DNAs with oligonucleotide primers corresponding to their flanking sequences, Dr. McBride and his colleagues have identified in chromosome 22 six dispersed highly polymorphic multisatellite markers which were subsequently used for linkage analysis in CEPH and NF2 families.

Dr. Singer and her colleagues are characterizing p40, the protein encoded in ORF1 of the L1Hs (human) retrotransposon. The polypeptide is cytoplasmic, phosphorylated, and includes a leucine zipper region capable, in principle, of forming a coiled-coil dimer. Complexes of p40 have been detected which could include homo- or heteromultimers. Extending earlier results on *cis* acting motifs important for transcriptional regulation of L1Hs in human teratocarcinoma cells, these investigators have identified four protein-binding sites in the 5'UTR.

DNA REPLICATION

Studies of plasmid maintenance by the group of Drs. Yarmolinsky and Chattoraj have focused on the molecular events involved in replication and partition of the archetypical low-copy-number plasmid, P1. These studies have established that the function of the bacterial heat shock proteins, DnaJ, DnaK and GrpE, in promoting P1 plasmid replication is limited to an activation of the plasmid-encoded initiator, RepA, for binding to the P1 origin, that this activation involves the refolding of monomeric RepA protein, that there is a cooperative interaction of RepA with the bacterial initiator, DnaA, and that it is the latter protein that appears responsible for a strand opening which triggers initiation of replication. Studies of partition have challenged established concepts about the structure and function of the P1 partition module and provided new tools which will enable them to obtain information that will be needed to construct a coherent model of partition.

Dr. Michael Lichten is studying the mechanism and regulation of meiotic recombination in the yeast *Saccharomyces cerevisiae*. His group has examined the relationship between meiotic recombination and meiosis-induced double-strand DNA breaks, which have been suggested as initiators of meiotic recombination. They have shown that chromosome context is important in determining the site and frequency of these breaks. Break sites are determined primarily by elements of chromatin structure, rather than by recognition of particular sequences. They have also shown that chromosome elements can act over distances of as great as 20 kb to modulate the frequency of breaks and the level of recombination at a given site. Dr. Lichten's laboratory is currently engaged in the isolation of the nuclease responsible for these breaks, and is also engaged in the isolation and characterization of DNA intermediates in meiotic recombination.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00366-21 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure and Expression of Endogenous Retroviral Elements

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

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

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Biosynthesis Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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

B

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

We have completed a study of the DNA binding properties of a ubiquitous nuclear protein isolated in our laboratory as a transcription factor (EBP-80) for mouse intracisternal A-particle LTRs and subsequently found to be the same as a previously known protein (Ku) first detected by antibodies from certain autoimmune patients. Ku is known to bind to the ends of duplex DNA. Our study confirms that EBP/Ku binds with high affinity (Kd's of 10-40 pM) to blunt-ended duplex DNA. However, it binds equally strongly to duplex DNA in which the strands are connected at either end by hairpin loops (i.e., there are no free strand ends), and to constructs consisting of short stretches of duplex DNA with one or more single strand extensions. EBP/Ku also binds to circular duplex molecules containing a short single-stranded region or a double-stranded region of non-homology (bubble), but does not bind to the corresponding construct consisting entirely of duplex DNA. We conclude that EBP/Ku recognizes single-to-double strand transitions in DNA. Binding to regions of strand separation could be related to the transcriptional enhancement observed by us and others, as well as to a possible role in DNA replication. The functional role of this protein is currently under study. We earlier identified a discrete and highly related subpopulation of IAP elements (LS elements) that are transcriptionally active in normal mouse B lymphocytes and thymus cells. Oligonucleotide probes specific for the three LS subfamilies each hybridize with a restricted number of fragments in gel-fractionated genomic restriction digests. We have found that each probe detects multiple polymorphisms between different strains of inbred mice and thus can be used for chromosome mapping of IAP elements. The LS probes are already in active use in this and other laboratories as multilocus probes for genomic mapping, linkage analysis and strain identification in inbred mice (see Annual Report, Dr. K.K. Lueders).

Project Description

Major Findings:

A. Transacting cellular factors for IAP expression (Dr. M. Falzon)

In previous reports we described the purification and characterization of a transcription factor, designated EBP-80, isolated from transformed human cells by affinity chromatography on an oligonucleotide representing a regulatory sequence in IAP long terminal repeats (LTRs). In the presence of non-specific competitor such as poly dC:dI, EBP-80 binds preferentially to sequences with homology to the SV40 enhancer core. The protein enhances transcription in vitro from the IAP LTR; enhancement is methylation-sensitive providing the template is presented in closed circular form. Transfection studies indicate that EBP-80 stimulation of LTR promoter activity in vivo is also sensitive to site-specific methylation. EBP-80 may mediate in part the known inhibitory effect of LTR methylation on IAP expression.

As reported last year, EBP-80 was found by micro-amino acid sequencing to be indistinguishable from Ku, a heterodimeric nuclear protein first detected by antibodies from autoimmune patients. Ku (and EBP-80) consists of 85 and 70 kDa subunits present in equimolar amounts. A number of laboratories have shown that the Ku protein complex binds to the free ends of double-stranded DNA.

In a study continued from last year and recently completed, we have examined the binding characteristics of EBP/Ku in some detail. The protein was shown to bind single-stranded DNA with low affinity. Binding to double-stranded DNA depends on the length of the duplex, being optimal with oligomers of 24 or more bp. The protein/DNA complexes are very stable, with Kd's of 10-40 pM and off-times measured in hours. EBP/Ku prefers A/T- to G/C-rich ends. It binds with high affinity to blunt-end duplex DNA, to duplex DNA in which the two strands are connected at either end by hairpin turns, and to constructs consisting of short stretches of double-stranded DNA with one or more single-strand extensions. EBP/Ku also binds to circular duplex molecules containing a short single-stranded region or a double-stranded region of non-homology (bubble), but does not bind to the corresponding closed circular construct made up entirely of duplex DNA. We have therefore concluded that EBP/Ku recognizes single- to double-strand transitions in DNA. We are currently investigating whether the protein can recognize other perturbations in the regular structure of B-form DNA, e.g., Z-configuration or bends at junctions of A-rich runs. Binding to regions of strand separation or other conformational alterations could be related to the transcriptional enhancement observed by us and others, as well as to a possible role in DNA replication. Studies on the intranuclear localization and function of EBP/Ku are continuing.

B. Selective activation of IAP proviral elements in mouse lymphocytes

The IAP gene family is a closely related set of endogenous proviral-like sequences present in the genome of all inbred mouse strains. Approximately 2000 copies per genome are dispersed over the entire chromosome complement. IAP elements are a potentially important source of marker loci for the mouse genome, as well as indicators of local chromatin activity. However, because of the

large number of homologous genomic copies, it has been difficult to resolve individual proviral elements on conventional Southern blots hybridized with IAP-derived probes.

Last year, we (J. Mietz, J. Fewell and E. Kuff) described the identification of a discrete and highly related subpopulation of IAP elements characterized by their transcriptional activation in normal BALB/c lymphocytes and in the thymus of a number of inbred strains of mice. This lymphocyte-specific (LS) subset of IAP elements all shared a distinctive U3 regulatory region in their LTRs. However, small variations within a nine-nucleotide motif in their R regions divided the related elements into three unique subfamilies.

In a study currently in press, oligonucleotide probes specific for the three LS sub-families were used to examine the distribution of these elements in the genomic DNA of a number of inbred strains. Each of the three probes hybridized with a restricted number of fragments in electrophoretically fractionated restriction digests, enabling an uneqivocal comparison of the restriction patterns given by the various strains. Each oligonucleotide probe identified multiple polymorphisms between the different strains. The distribution of polymorphic restriction fragments among the standard CxB (C57BL/b x BALB/c) set of recombinant inbreds was determined in order to demonstrate the feasibility of using these probes for chromosome mapping. These and several other subset-specific probes subsequently identified are already providing an important group of multilocus markers for genomic mapping, linkage analysis and strain identification in inbred mice (see Annual Report of Dr. K. Lueders). LS elements are also found in distantly related mouse species, providing new markers for evolutionary studies.

Publications:

Mietz JA, Fewell JW, Kuff EL. Selective activation of a discrete family of endogenous proviral elements in normal BALB/c lymphocytes, Mol Cell Biol 1992;12:220-8.

Mietz JA, Kuff EL. Intracisternal A-particle-specific oligo nucleotides provide multilocus probes for genetic linkage studies in the mouse, Mammalian Genome 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00945-19 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

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

B. Peterkofsky	Chief, Biological Interactions Section	LB	NCI
A. Gosiewska	Visiting Fellow	LB	NCI
D. Kipp	IPA	LB	NCI
S. Wilson	Biologist	LB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Biological Interactions Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3	1

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

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

Our previous studies provided evidence that guinea pigs that are vitamin C deficient and those that have been fasted, but supplemented with vitamin C, are equivalent with respect to the mechanisms responsible for decreased collagen and proteoglycan synthesis. Sera from both groups contain a circulating factor that inhibits these functions and DNA synthesis in cultured connective tissue cells and inhibition is reversed by insulin-like growth factor (IGF)-I. The presence of the inhibitor in sera was associated with an increase in low molecular weight IGF binding proteins (IGFBPs) that can inhibit binding of IGF-I to its cellular receptor. We have identified them as IGFBP-1 and IGFBP-2 with antibodies that were produced using peptides with conserved IGFBP-2 sequences or native IGFBP-1 purified from rat hepatoma cells, as antigens. The mRNAs for these IGFBPs in liver are increased early during fasting and soon after weight loss commences during vitamin C deficiency, which is in agreement with the appearance of these IGFBPs in sera, as measured on ligand blots with [125I]IGF-I. The increase in IGFBP mRNA concentrations occurs prior to or concommitant with the time when type I collagen mRNA concentrations in bone and skin begin to decrease during the nutritional deficiencies. In contrast, the decline in IGF-I mRNA concentrations occurs more slowly than the changes in the mRNAs for collagen and the IGFBPs. These results are compatible with an in vivo role for IGFBPs as inhibitors of collagen synthesis during fasting and vitamin C deficiency in guinea pigs, independently of the level of circulating IGF-I.

Project Description

Objectives:

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

Major Findings:

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

A. Background

Our previous results suggested that ascorbate-deficient and fasted (ascorbate-supplemented) guinea pigs are equivalent with respect to the mechanisms by which collagen and proteoglycan synthesis are decreased in connective tissues. Furthermore, sera from these animals could transmit the defects in extracellular matrix synthesis to cultured connective tissue cells in the presence of ascorbate through the action of an inhibitor. These sera also inhibited the stimulation of DNA synthesis in quiescent 3T3 cells by normal guinea pig serum. 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 suggested that the inhibitor might be an IGF-I binding protein (IGFBP). Analysis of sera by cross-linking/SDS-PAGE and gel filtration showed that two IGFBPs with unoccupied binding sites increased during scurvy and fasting. Ligand blotting after SDS/PAGE showed that the major carrier of IGFs in serum, IGFBP3, was present in all guinea pig serum, but in sera from guinea pigs on a vitamin C-free diet for 4 weeks or fasted for 4 days, there were two IGFBPs, 30 and 35 kDa, that were dramatically increased. After gel filtration of sera, inhibition of binding of IGF-I to its receptor on 3T3 cells coincided with IGFBP activity. These and other data supported the concept that the inhibitor of IGF-I dependent functions consisted of one or both of the IGF binding proteins.

B. Preparation of Antibodies to IGFBPs

We previously identified one of the IGF binding proteins, a 30-kDa protein, as IGFBP-1 using an antibody to the human protein that was obtained from another laboratory. In order to confirm the identify the putative IGFBP-2 induced during scurvy and fasting, it was necessary to prepare antibody for this protein. Additional antibody for IGFBP-1 also was required since we planned to carry out experiments in which guinea pig serum would be depleted of IGFBPs using antibodies and then tested for reversal of inhibition of collagen synthesis in cultured cells. Since the amount of the IGFBPs in fasted or scorbutic guinea pig serum was estimated to be quite low and there is immunological cross-reactivity between species for both IGFBP-1 and 2, more convenient sources of antigens were used.

1. Identification of the 35-kDa IGFBP

Based on the greater affinity of the 35 kDa binding protein for IGF-II compared to IGF-I, and its size, it seemed likely that it was IGFBP-2. We had three peptides synthesized that corresponded to highly conserved regions of the molecule, including the N and C-terminals and a hydrophilic sequence from the central region. The peptides were coupled to keyhole limpet hemocyanin and rabbits were immunized with each. All three produced antibody, measured by ELISA assay with the peptides as antigen. On Western blots, all three antibodies reacted with authentic IGFBP-2 and with the 35 kDa binding protein in fasted and scorbutic guinea pig sera, establishing its identity as IGFBP-2.

2. Purification of IGFBP1 and preparation of antibody

A rat hepatoma cell line, H-35, produces a single binding protein, IGFBP1, and its synthesis can be induced 10-20 times by treatment with the glucocorticoid steroid dexamethasone. The protein was purified from 4 liters of serum-free conditioned medium from H-35 cells treated with dexamethone. Because of the extent of induction by the steroid, relatively few steps were required for purification with a high yield. The medium was concentrated and partially purified by ammonium sulfate precipitation and the binding proteins were fractionated sequentially on a Sephacryl 200HR column and then a Bio-Rad Q cartridge anion exchanger. Two species of BP-1 were obtained that eluted at 0.1 M and 0.15 M salt. The first species, QA, gave a single band corresponding to 31 kDa when analyzed on SDS/PAGE with silver staining, which is the size of rat IGFBP-1. The protein also reacted in the ligand blot assay with [125I]IGF-I and in a Western blot with anti-human IGFBP-1. The binding protein in the 0.15 M salt fraction (QB) reacted similarly but had a trace contaminant in the high molecular weight region on SDS/PAGE so it was refractionated on S200HR. Both proteins were used as antigens to immunize rabbits and both produced antibodies that reacted with IGFBP-1 in guinea pig sera.

C. Removal of IGFBPs from Guinea Pig Sera

1. IGFBP-1

Preliminary experiments showed that antiBP-1(QA) effectively immunoprecipitated IGFBP-1 in fasted guinea serum. The IgG fraction of the antiserum was purified on a protein A column. Normal and fasted guinea pig sera were incubated with antibody and passed through a protein A column to remove the IgG-binding protein complex. Analysis of the non-adsorbed fraction by a ligand blot confirmed that essentially all of the IGFBP-1 had been removed from the sera, leaving IGFBP-3, the major IGF-I carrier, in the normal serum and IGFBP-3 and IGFBP-2 in the fasted serum. Experiments are under way to determine whether the IGFBP-1-depleted, fasted serum will still inhibit collagen synthesis in cultured cells compared to normal guinea pig serum treated with the antibody.

2. IGFBP-2

Although the anti-BP-2 peptide antibodies were useful for identifying the 35-kDa binding protein on Western blots, they were not very effective at immunoprecipitating the protein. Therefore, a source that yields sufficient native IGFBP-2 is

being evaluated. The protein will be purified by methods similar to those used for purifying IGFBP-1 and rabbits will be immunized with the purified protein. Guinea pig sera will be depleted of IGFBP-2 using the antibody and they will be tested for inhibitor activity as described above.

D. Relationship of IGFBPs to In Vivo Expression of Collagen

This aspect of the project was designed to determine whether the temporal expression of the IGFBPs during nutritional deprivation in vivo was compatible with their proposed role as inhibitors of collagen synthesis. This was approached by measuring mRNA levels for IGF-I and IGFBP-1 and -2 in liver, as well as mRNA for type I collagen in several connective tissues during vitamin C deficiency and fasting.

1. Fasting

Tissues were removed from guinea pigs that were fasted for 1, 2, 3 and 4 days and from control fed animals and RNA was isolated. The results showed that mRNA for the α1(I) subunit of type I collagen decreased in skin and bone, but the regulation was not coordinate. In skin, mRNA levels had decreased to 40% of control values after 24 h while the decrease in bone lagged behind by about 24 h. These changes preceded a decline in IGF-I mRNA levels. In contrast, concentrations of mRNAs for IGFBP 1 and 2 were already dramatically increased after 24 h of fasting.

2. Vitamin C-deficiency

Tissues were removed from guinea pigs that exhibited varying extents of weight loss during the third and fourth weeks of vitamin C deficiency and analysis of mRNAs was carried out as described above. Results were similar to those from fasted animals in that the decline in type I collagen mRNA in skin commenced as soon as weight loss began while the decline was slower in bone. mRNA concentrations for the IGFBPs in liver were already increased at 5% weight loss while IGF-I decreased only after guinea pigs had lost about 20% or more of their body weight, which was beyond the point when collagen mRNA levels began to decline. The pattern of changes in the expression of mRNAs for the IGFBPs relative to collagen mRNA during the two nutritional deficiency states is compatible with the proposed role of the IGFBPs as inhibitors of collagen synthesis. Furthermore, the delayed decline in IGF-I mRNA levels is in agreement with our previous observations that some scorbutic guinea pig sera with normal IGF-I concentrations nevertheless contained inhibitor.

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

A. Background

Early work had shown that wound healing was impaired during vitamin C deficiency. It was suggested that this resulted from defective collagen formation due to the requirement for ascorbate in proline hydroxylation. Based on our recent work, however, there are only moderate effects of vitamin C deficiency on proline hydroxylation in non-repair connective tissues. Effects on hydroxylation do not appear to be involved in the decreased collagen synthesis in these

in these tissues that occurs during the period of weight loss in the third and fourth week on a vitamin C-free diet, which we refer to as phase II of scurvy. Phase I of scurvy consists of the first two weeks on the vitamin C-free diet when the guinea pigs grow as well as controls although vitamin C levels are decreased by about 90% after one week. In past studies where wounds were initiated and analyzed during phase I, wound healing was defective and concentrations of hydroxyproline were markedly decreased. Those studies, however, could not distinguish whether collagen synthesis or inhibition of proline hydroxylation was responsible for the decrease. This project was inititiated to determine whether defective collagen synthesis or proline hydroxylation occurred in wound tissue during phase I of scurvy when collagen synthesis in non-repair connective tissues is not affected.

B. Collagen Synthesis and Proline Hydroxylation in Wound Tissue

Guinea pigs were placed on a vitamin C-free diet with or without oral ascorbate supplementation. After 7 days, when ascorbate levels in tissues are decreased, the animals were anesthesized and two 1 cm incisions were made on the back. Vinyl sponge squares were implanted under the skin to collect granulation tissue and the wound was closed with a wound clip. After 10 days (17 days on the diet), the implants were removed and collagen synthesis and proline hydroxylation in one implant was measured after a short-term culture with radioactive proline. At the time of removal of the implants, the vitamin C-deficient animals were gaining weight at about the same rate as control animals but healing of the incision was incomplete compared to the wound in the normal guinea pigs. Preliminary analyses indicate that in non-repair tissues such as cartilage and bone, the relative rate of collagen synthesis was not greatly affected, as expected. In the implants from scorbutic guinea pigs, collagen synthesis was reduced by about 40%, but surprisingly, the percentage of proline hydroxylation in collagen was only about 40% lower than in the implant in control animals. Our results suggest that defective wound healing occurs by a different mechanism than that leading to inhibition of collagen synthesis in phase II of scurvy.

III. Expression of Extracellular Matrix Genes in In Vitro and In Vivo Aged Human Skin Fibroblasts

The regulation of the synthesis of procollagen and other extracellular matrix components was examined in human skin fibroblasts obtained from donors of various ages, from fetal to 80 years old (in vivo aged), and in fetal fibroblasts at varying passage levels (in vitro aged). Growth rates and saturation densities of fibroblasts decreased with increasing age of the donor and after passage 20 of fetal fibroblasts. The rates of collagen and proteoglycan synthesis also decreased during both types of aging to about 10-25% of the rate in early passage fetal fibroblasts, while the synthesis of total noncollagenous proteins was not greatly affected. Decreased collagen synthesis in both types of aging was correlated with lower steady state levels of mRNAs for the two subunits of type I procollagen mRNA, although their regulation was not coordinate. Type III collagen mRNA levels also declined in both types of aging. The concentration of fibronectin mRNA also decreased during in vitro aging but more rapidly than the collagen mRNAs, while it was similar or higher in fibroblasts from 51-80-year-old donors than in early passage fetal fibroblasts. This study suggests that the decreased synthesis of procollagen and proteoglycans in

in vivo aged fibroblasts represents changes that are responsible for intrinsic degenerative changes that occur in human skin during aging. Furthermore, although *in vitro* and *in vivo* aging were similar in many respects, they were not equivalent, as evidenced by the differences in regulation of fibronectin expression.

Publications:

Peterkofsky B. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy, Am J Clin Nutr 1991;54:1135S-40S.

Peterkofsky B, Prather W. A post-translational modification, unrelated to hydroxylation, in the collagenous domain of nonhelical proα2(I) procollagen chains secreted by chemically transformed hamster fibroblasts, J Biol Chem 1992;267:5388-95.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05202-25 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Isolation, Fractionation and Characterization of Native Nucleoproteins

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

O.W. McBride	Chief, Cellular Regulation Section	LB	NCI
H.-F. Yi	Visiting Fellow	LB	NCI
M. Hawkins	IPA Investigator	LB	NCI
D. Sabourin	Staff Fellow	LB	NCI
M.G. Wang	Guest Researcher	LB	NCI
J. Clark	Laboratory Worker	LB	NCI

COOPERATING UNITS (if any) NICHHD: R. Klausner; NIAMSD: P. Steinert, S.J. Bale; NCI: K. Kelly, D. Parry, P. Howley, S. Rosenberg, F. Gonzalez, F. Kaye, L. Litou; NIMH: S. Young, S.J. Lolait, T.B. Usdin, M.J. Brownstein; A. Arnold (Harvard); N. Nussmeier (UCLA); C. Hill (Vanderbilt); R. Pirtle (U. Texas); E. Strehler (Zurich)

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Cellular Regulation Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4	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.)

Human genetics research in this laboratory falls within three general categories. The first involves the chromosomal localization of cloned sequences including genes and tumor breakpoints by Southern analysis of human-rodent somatic cell hybrids, in situ hybridization, and genetic linkage analysis in 40 large CEPH families. About 20 additional cloned genes have been mapped during the past year by one or more of these methods. Of special note are localization of the Vasopressin V2 receptor to Xq27-q28 in the same region as the nephrogenic diabetes insipidus (NDI) locus and mapping of a plasma membrane Ca ATPase (PMCA2) to the 3p25-p26 boundary which contain the locus for von Hippel Lindau syndrome (VHL). The second involves the use of linkage analysis for mapping and identifying specific tumor susceptibility, and other disease, genes in affected families. Collaboration with Drs. D. Parry and R. Eldridge continues in mapping the neurofibromatosis 2 (NF2) gene, and two new microsatellite markers near this locus were used to localize points of recombination near the NF2 gene in individuals from two families. Genes responsible for several genetic skin diseases are being mapped in collaboration with Drs. P. Steinert and S.J. Bale. Epideromolytic hyperkeratosis (EHK) was found to cosegregate with the type II keratin gene cluster on chromosome 12q and probably involves the keratin 1 gene. Studies are in progress with Epidermolysis vulgaris, Darier's disease, and Basal nevus syndrome. The third area involves cloning and sequencing regions on chromosomes 12 and 22 containing polymorphic long repeats of trinucleotides to use in constructing high resolution genetic and physical maps of these chromosomes for mapping NF2 and other disease genes. Oligonucleotide primers are synthesized to permit PCR amplification of genomic DNAs. Most tracts have been found to be multiallelic and highly polymorphic, and some were used for linkage analysis in CEPH and NF2 families. Most of the $(AAT)_n$ tracts immediately flank the 3' end of ALU sequences (i.e. in poly A flanks) but this has not precluded use of these tracts.

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 and other diseases.

Major Findings:

A panel of human-rodent somatic cell hybrids previously isolated and characterized in this laboratory continues to be used for chromosomal mapping of cloned human genes in collaboration with investigators at NIH and elsewhere. Some of the genes mapped by this method (and collaborators) during the past year have included trichohyalin and transglutaminase 1 (P. Steinert, NIAMSD), cyclin D3 and an ovarian tumor translocation breakpoint on 11q (A. Arnold, Harvard), four transcriptional activators from a steroid hormone receptor superfamily (M. Nussmeier, UCLA), carboxyesterase and a chlofibrate receptor (F. Gonzalez, NCI), an antigen (MAGE) recognized by cytolytic T-lymphocytes in human melanomas (R. Zakut & S. Rosenberg, NCI), vasopressin V2 receptor (S.J. Lolait & M.J. Brownstein, NIMH), a dopamine transporter (T.B. Usdin & M.J. Brownstein) retinoblastoma binding protein (F. Kaye, NCI), genes for two Golgi proteins (R. Klausner, NICHHD), two transcriptional regulatory genes (S. Young, NIMH; P. Howley, NCI), seloprotein P (C. Hill, Vanderbilt), a $tRNA^{Leu}$ pseudogene (R. Pirtle, U. Texas), and a T-cell leukemia putative breakpoint (L. Litou, NCI).

In situ hybridization with biotinylated DNA probes, genetic linkage analysis in CEPH families, and analysis of cell hybrids containing specific chromosome breaks have been used to regionally localize cloned genes on chromosomes. Trichohyalin has been localized to 1q21 by *in situ* hybridization and genes for three other epidermal proteins (i.e., loricrin, profilaggrin, and involucrin) have the same physical location. Linkage analysis indicates that profilaggrin and loricrin are closely linked (about 2 megabase interval), but one recombinant has been found in the CEPH pedigrees. Linkage analysis is now in progress involving all four genes. Results of *in situ* hybridization localize a plasma membrane Ca ATPase (PMCA 2) to the von Hippel Lindau region (border of 3p25-3p26) and transglutaminase 1 and MAGE were localized to a pericentric region (14q11) and telomeric region (Xq27-q28), respectively. Preliminary linkage analysis using a dinucleotide microsatellite sequence within the TGM 1 gene confirms the result and will allow the gene to be ordered within a cluster of highly informative loci (at 14q11-q12) which we have previously studied. Linkage analysis also has been used for fine localization of the chlofibrate receptor, the ovarian tumor breakpoint, a gene for a ras-related protein on 8q, and three members of the steroid hormone receptor superfamily. A highly polymorphic VNTR detected with the dopamine transporter cDNA will be used to further localize this gene.

MAGE cDNA or genomic probes detect a family of at least 4 sequences, consistent with previous reports from another laboratory. Most, or all, of these sequences appear to be located on Xq. However, examination of DNAs from cells containing only fragments of human X (resulting from chromosome mediated gene transfer) indicates the sequences are present at more than a single locus. In contrast, only a single locus was detected by in situ hybridization with a biotinylated cosmid DNA probe. Additional loci might not be detected by this method even if they were separated by distances within the resolution of the fluorescence microscope.

Oligonucleotide probes have been synthesized which can detect all potential trinucleotide repeat sequences, and each has a 5' extension containing a BamHI restriction site. Complete digest (EcoRI) chromosome 22 and chromosome 12 libraries (LA22NS03 and LA 12NS01) have been screened with 32P-end labeled $(AAT)_9$ probe, and positive plaques isolated at a frequency of about 0.5%. After plaque purification, minipreps were used for PCR amplification using "internal" $(AAT)_9$ or $(ATT)_9$ primers with lambda phage primers from each flank of the insert. After EcoRI/BamHI double digestion, the PCR products were subcloned into a pGEM plasmid vector and minipreps isolated. A universal primer flanking the BamHI site was used for DNA sequencing across the trinucleotide repeat and through about 150-200 flanking nucleotides. Unique oligonucleotide primers within each flank were synthesized and used for PCR amplification of genomic DNAs to detect length polymorphisms by denaturing polyacrylamide gel electrophoresis and autoradiography. DNAs from the CEPH families were used to determine heterozygosities and PICs and for linkage analysis with other markers. This system has generated six highly polymorphic (most with PIC about 0.7) multiallelic microsatellite markers on chromosome 22, and additional markers from both chromosomes have not been fully examined yet. The markers are dispersed and about 80% immediately flank the 3'-end of an ALU sequence and presumably arose by a single A → T mutation within the ALU 3' polyA tract followed by amplification. It has been possible to construct primers from divergent sequences within the ALU, or from more distal unique sequence beyond the ALU, for use in linkage analysis. All members of the CEPH families have been examined with 5 of these markers, and 3 have been ordered on chromosome 22 by multipoint linkage analysis. Probes for some of the other trinucleotide repeats are being used for screening to determine whether they are equally frequent and equally polymorphic and whether they also arise in the polyA tracts of ALU sequences and other retrotransposons.

Collaborative studies with Drs. Roswell Eldridge and Dilys Parry of the Interinstitute Genetics group have confirmed the location of the gene for neurofibromatosis type 2 (bilateral acoustic schwannomas) on chromosome 22 in two additional families, but the most probable location is still reasonably large (i.e. about 15-20 cM). Two of the trinucleotide repeat markers recently isolated are located within this interval and allow the localization of the NF2 gene to be further narrowed. Additional markers of this type are being tested in these two families with the objective of narrowing the locus to 1-2 cM prior to efforts to clone the gene. Collaborative studies with Drs. P. Steinert and S.J. Bale involve identifying the disease gene in a number of hereditary skin diseases. We have immortalized lymphoid cell lines and isolated DNA from families with several different diseases including Epidermolytic Hyperkeratosis (EHK), Basal nevus syndrome, Epidermolysis Vulgaris, and Darier's disease. Both

of our labs are typing these DNAs with polymorphic markers within, and closely linked to, a variety of candidate genes. Close linkage of the EHK gene to the keratin type II cluster on chromosome 12 has been shown in one family by the combined studies in both labs. Preliminary studies by Steinert et al. suggest that keratin 1 is the disease gene in this family. Additional candidate genes also are being cloned, mapped, and polymorphic markers identified to facilitate the identification of the defect in these other skin diseases.

As a CEPH collaborator assigned to construct a genetic linkage map of human chromosome 12 from all information in the CEPH database version 5, a preliminary map has been constructed by multipoint linkage analysis containing 16 loci spanning nearly the entire chromosome and a distance of 186 cM assuming no sex difference in recombination fraction. This map has been submitted to CEPH. Some errors in the database have been detected and corrected. Regions containing a sparsity of markers and relatively uninformative markers have been noted, and efforts will be made to supplement the data in these regions prior to preparation of the final map.

Publications:

Mitchell A, Bale AE, Wang-ge M, Yi HF, White R, Pirtle RM, McBride OW. Localization of a DNA segment encompassing four tRNA genes to human chromosome 14q11 and its use as an anchor locus for linkage analysis, Genomics 1991;11:1063-70.

Dodge GR, Kovalszky I, Chu M-L, Hasell JR, McBride OW, Yi HF, Iozzo RV. Heparin sulfate proteoglycan of human colon: partial molecular cloning, cellular expression, and mapping of the gene (HSPG2) to the short arm of human chromosome 1, Genomics 1991;10:673-80.

Bale AE, Mitchell AL, Gonzalez FJ, McBride OW. Localization of CYP2F1 by multipoint linkage analysis and pulsed-field gel electrophoresis, Genomics 1991;10:284-6.

Zahnow CA, Yi HF, McBride OW, Joseph DR. Cloning of the cDNA encoding human histidine decarbosylase from an erythroleukemia cell line and mapping of the gene locus to chromosome 15, J DNA Sequencing and Mapping 1991;1:395-400.

Chin H, Kozak CA, Kim HL, Mock B, McBride OW. A brain L-type calcium channel α1 subunit gene (CCHL1A2) maps to mouse chromosome 14 and human chromosome 3, Genomics 1991;11:914-9.

Dodge GR, Kovalszky I, McBride OW, Yi HF, Chu ML, Saitta B, Stokes DG, Iozzo RV. Human clathrin heavy chain (CLTC): partial molecular cloning, expression, and mapping of the gene to human chromosome 17q11-qter. Genomics 1991;11:174-8.

Craig IW, McBride OW. Report of the committee on the genetic constitution of chromosome 12 (HGM11), Cytogenet Cell Genet 1991;58:555-79.

Gailani MR, Bale SJ, Leffell DJ, DiGiovanna JJ, Peck GL, Poliak S, Drum MA, Pastakia B, McBride OW, Kase R., Greene M, Mulvihill JJ, Bale AE. Developmental defects in Gorlin Syndrome related to a putative tumor suppressor gene on chromosome 9, Cell 1992;69:111-7.

Lolait SJ, O'Carroll AM, McBride OW, Konig M, Morel A, Brownstein MJ. Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus, Nature 1992;357:336-9.

Kim IG, McBride OW, Wang M, Kim SY, Idler WW, Steinert PM. Structure and organization of the human transglutaminase 1 gene, J Biol Chem 1992;267:7710-7.

Korge BP, Gan SQ, McBride OW, Mischke D, Steinert PM. Extensive size polymorphisms of the human keratin 10 chain resides in the C-terminal V2 subdomain due to variable numbers and sizes of glycine loops, Proc Natl Acad Sci USA 1992;89:910-4.

Yoneda K, McBride OW, Korge BP, Kim IG, Steinert PM. The cornified cell envelope: loricrin and transglutaminases, Jap J Dermatol 1992; in press.

Fleischhauer K, McBride OW, DiSanto JP, Ozato K, Yang SY. Cloning and chromosome mapping of human Retinoid X Receptor B: selective amino acid sequence conservation of a nuclear hormone receptor in mammals, Hum Genet 1992; in press.

McBride OW. Gene mapping by genetic linkage and somatic cell hybrid analysis. In: Kirsch IL, ed. Causes and Consequences of Chromosomal Aberrations. Boca Raton, FL: CRC Press Inc., 1992; in press.

Motokura HF, Yi HF, Kronenberg HM, McBride OW, Arnold A. Assignment of the human Cyclin D3 gene (CCND3) to chromosome 6p-q13, Cytogenet Cell Genet 1992; in press.

Yoneda K, Hohl D, McBride OW, Wang M, Cehrs KU, Idler WW, Steinert PM. The human loricrin gene, J Biol Chem 1992; in press.

Park JS, Lucthy JD, Wang MG, Fargnoli J, Fornace AJ, McBride OW, Holbrook NJ. Isolation, characterization and chromosomal localization of the human GADD153 gene, Gene 1992; in press.

Mitchell A, Bale AE, Lee BJ, Hatfield D, Harley H, Rundle SA, Fan YS, Fukushima Y, Shows TB, McBride OW. Regional localization of the selenocysteine tRNA gene (TRSP) on human chromosome 19, Cytogenet Cell Genet 1992; in press.

Compton JG, DiGiovanna JJ, Santucci SK, Kearns KS, Amos CI, Abangan D, Korge BP, McBride OW, Steinert PM, Bale SJ. Epidermolytic Hyperkeratosis completely cosegregates with the type II keratin gene cluster on chromosome 12q, Nature Genetics 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05203-24 LB

PERIOD COVERED: October 1, 1991 to September 30, 1992

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

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

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

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Biochemistry, DCBDC

SECTION: Biosynthesis Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.50	2.50	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (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 has a long-standing interest in immunochemical methods for cell separation, and in developing engineered macromolecular reagents for use in studying relatively low affinity interactions (such as those between MHC molecules and T cell receptors). Because balanced production of naturally multimeric proteins (such as antibodies) can be facilitated by engineering them as single chain molecules, we have engineered soluble recombinant single chain Class I MHC (MHC I) molecules. These recombinant molecules are useful for studying protein folding and assembly, and may serve as precursors for toxin-conjugates and T cell vaccines.

During this year, our laboratory has prepared several recombinant soluble single chain MHC I molecules, which have their β2-microglobulin (β2m) domain covalently linked to the heavy chain. We have characterized two of them extensively. One molecule has wild type amino acid sequences. The other is a mutant molecule which lacks three salt bridges in the peptide-binding domains. The mutant molecule is not thermally stable and is secreted at 27° but not at 37°. Both molecules contain endogenous peptides. This indicates that they have folded correctly, and suggests that a β2m-free heavy chain/peptide complex is not an obligate intermediate in assembly. When pulsed with an antigenic peptide from the HIV gp120 envelope protein, both molecules can present it to an H-2D^d restricted antigen-specific T hybridoma. This indicated that the recombinant molecules are biologically active and have bound the peptide in the same configuration as in the wild-type two chain MHC I molecules. Because the single chain molecule, despite a tethered β2m, retains sufficient flexibility to be loaded in vitro with antigenic peptides, it may be useful for structural studies, and as an intermediate in the formation of toxin-conjugates and T cell vaccines.

Project Description

Objectives:

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

Major Findings:

In collaboration with Dr. David Margulies and his colleagues, we have engineered and characterized two recombinant soluble single-chain class I (MHC I) molecules that have a covalently linked non-dissociable β2m. These recombinant molecules are expressed and secreted by transfected mammalian "L" cells. (The natural MHC I molecules which have noncovalently bound dissociable β2m play an essential role in some immune responses. When cells are infected with viruses or other pathogens, the MHC I molecules bind antigenic peptides from endogenously synthesized pathogen proteins, and present them to T cell receptors. In the absence of infection, the MHC I molecules bind endogenous self peptides to which the T cell population is tolerant, except in some autoimmune conditions.)
A. One single chain molecule has wild-type amino acid sequences, and the other is a mutant molecule that lacks three salt bridges in the peptide binding domains.

B. The mutant molecule is not thermally stable, and is secreted at 27° but not at 37°.

C. Both mutant and wild-type single chain molecules contain endogenous peptides. This indicates that they have folded correctly, and suggests that a β2m-free heavy chain/peptide complex is not an obligate intermediate in assembly. (The order of assembly of MHC I molecules is unknown. The heavy chain might first bind to β2m and then bind peptide, or alternatively, the heavy chain might first bind peptide, and then bind β2m).

D. Both single chain molecules can be pulsed with an antigenic peptide from the HIV gp120 envelope protein. They present that peptide to an H-2D^d restricted antigen-specific T cell hybridoma. This indicates that both recombinant molecules are biologically active and have bound the peptide in the same configuration as the wild-type two chain molecule. It further shows that the single chain molecule, despite a tethered β2m, retains sufficient flexibility to be loaded *in vitro* with antigenic peptides, and may thus be useful for structural studies, and as an intermediate in the formation of toxin-conjugates and T cell vaccines.

E. In collaboration with Dr. J. Schneck of Johns Hopkins University, our lab has also successfully transfected a recombinant MHC I heavy chain gene (H-2K^b) into a cell line that is defective in loading class MHC I molecules with peptides. The resultant two chain MHC I molecule could be found expressed on the surface of transfected cells at 27°, and when the cells were pulsed with

appropriate peptides, the molecules became stable at 37°. Such peptide-loaded cell surface MHC I molecules were used to show that certain monoclonal anti H-2 antibodies are specific for MHC I/peptide complexes, or (alternatively) that certain bound peptides can block the interaction of certain anti MHC I antibodies.

Publications:

Margulies DH, Boyd LF, Corr M, Hunziker RD, Khilko S, Kozlowski S, Mage M, Ribaudo RK. Class I MHC/peptide/ß2 microglobulin interactions: the basis of cytotoxic T cell recognition. In: Sitkovsky M, Henkart P, eds. Cytotoxic cells - generation, recognition, effector function, methods. Cambridge, MA 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05231-18 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation

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

C.B. Klee	Chief, Protein Biochemistry Section	LB	NCI
M.H. Krinks	Chemist	LB	NCI
A. Segelken	IRTA Fellow	LB	NCI
M.P. Strub	Visiting Fellow	LB	NCI
P. Stemmer	IRTA Fellow	LB	NCI
I. Myagkikh	US/Soviet Exchange Fellow	LB	NCI
H. Ren	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)* Dr. A. Bax, NIDDK; Dr. Craig Montell, Johns Hopkins University; Dr. S. Schreiber, Harvard University; Dr. S. Burakoff, Dana-Farber Cancer Institute, Boston; Dr. Ching Kung, University of Wisconsin; Dr. O. Wesley McBride, NCI; Dr. Eva Mezey, NINDB

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Protein Biochemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.00	4.50	0.50

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Work in this laboratory is aimed at elucidating the mechanism of stimulus-response coupling mediated by Ca^{2+} and calmodulin. The calmodulin-regulated protein phosphatase, calcineurin, is used as a model system. It was previously shown that the catalytic subunit of calcineurin, calcineurin A, is composed of a "catalytic domain" and a regulatory domain itself composed of an inhibitory and calmodulinbinding domain. The "catalytic domain" was shown this year to contain at least three subdomains, the catalytic center, the calcineurin B-binding site and the interaction site(s) for the immunosuppressant/immunophilin complexes. The calcineurin B-binding site has been identified and its interaction with calcineurin B, synthesized in E. coli, is being studied by multidimensional NMR in collaboration with Drs. Ad Bax and Jacob Anglister (NIDDK). Another collaborative project with Dr. Schreiber (Harvard University) has been initiated to identify the interaction site(s) of calcineurin with immunosuppressants. These studies were undertaken to establish the molecular basis of the role of calmodulin and calcineurin in the Ca^{2+}-mediated regulation of gene expression leading to T cell activation that was recently discovered by Dr. Schreiber and his colleagues. Another important development has resulted from a collaborative project with Drs. Bax and Ikura at NIH. This is the elucidation of the structure of the complex of calmodulin with a calmodulin-binding peptide. These studies helped to define structural requirements for calmodulin target protein interactions and revealed the importance of the flexibility of the central helix in this interaction.

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 roles of the two second messengers, Ca^{2+} and cAMP, in the regulation of cell function.

Major Findings:

Calmodulin and Calcium Regulation of Cellular Activity

A. Interaction of Calmodulin with Target Proteins and Peptides

In order to understand the mechanism of the Ca^{2+}-dependent activation of calmodulin-regulated enzymes, we have continued to study the Ca^{2+}-dependent interaction of these enzymes, or their calmodulin-binding domains, with calmodulin. The structure in solution of Ca^{2+}:calmodulin complexed with a synthetic peptide corresponding to the calmodulin-binding domain of skeletal muscle myosin light chain kinase has been determined using multidimensional NMR spectroscopy in collaboration with Dr. Bax and his colleagues. A disruption of the central helix, which forms a long flexible loop, enables the amino- and carboxyl-terminal halves of calmodulin to establish multiple contacts with the calmodulin-binding peptide. This structure implies that the requirements for a high affinity calmodulin-binding domain are accessibility and two hydrophobic residues, twelve residues apart, which anchor the peptide to the two halves of calmodulin. These limited requirements, which are shared by many calmodulin-binding domains, help to explain the different and at the same time competitive modes of interaction of calmodulin-regulated enzymes with calmodulin.

Several mutants, deficient in their ability to activate calcineurin, are being studied to identify differences in the mode of interaction of calmodulin with different enzymes. Two classes of Paramecium tetraurelium calmodulin mutants, deficient in their ability to stimulate either Ca^{2+}-dependent K^+ currents or Na^+ currents, have been isolated by Dr. Ching Kung and his colleagues. In collaboration with Dr. Kung's group, we have analyzed these mutants for their ability to stimulate calcineurin. Calmodulins mutated in the amino terminal half have a decreased Na^+ current and cannot fully stimulate calcineurin whereas calmodulins mutated in the carboxyl-terminal half have a decreased K^+ current and activate calcineurin as well as wild type calmodulin (C. Kung, K.-Y. Ling and M. Krinks).

The elucidation of the structure of the complex of calmodulin with calcineurin, a Ca^{2+}/calmodulin-stimulated protein phosphatase, is the next step in our study of the mechanism of action of calmodulin. The two subunits of calcineurin and calmodulin expressed in E. coli by Ren Hao will be used to crystallize the calcineurin/calmodulin complex. Calcineurin B, the Ca^{2+}-binding regulatory subunit of the enzyme, has been expressed in high yield. Its structure is being determined by multidimensional NMR in collaboration with Drs. Bax and Anglister to elucidate the structural basis for the different functional properties of calcineurin B and calmodulin, two related "EF-hand" Ca^{2+}-binding proteins.

B. Calcineurin: Structure-Function Relationships

This project deals with the decoding of the functional domains of calcineurin in order to understand how interaction with calmodulin results in enzyme activation. The catalytic subunit of calcineurin has previously been shown to consist of a catalytic domain, homologous to the catalytic subunits of many protein phosphatases, and a regulatory domain which consists of an autoinhibitory and calmodulin-binding domain. In the absence of calmodulin the regulatory domain, partially unfolded, is highly susceptible to proteolysis and easily severed from the rest of the molecule. Under these conditions the catalytic domain remains intact yielding a Ca^{2+}-stimulated enzyme which no longer needs calmodulin for activity. This proteolytic fragment of calcineurin A, calcineurin A-45, has a molecular weight of 45,000. It has preserved the calcineurin B-binding site which was shown to be located at the carboxyl-terminus of calcineurin A-45. The calcineurin A-45/calcineurin B complex was used by Paul Stemmer to study the calcineurin B-mediated Ca^{2+} regulation of calcineurin. The affinity of this complex for Ca^{2+} (k_{act}=5 x 10^{-8}M) is ten-fold higher than that of native calcineurin suggesting that in addition to its inhibitory effect on enzyme activity the regulatory domain of calcineurin also exerts a negative control on the affinity of calcineurin for Ca^{2+}. The ten-fold, calcineurin B-mediated, Ca^{2+} stimulation of calcineurin-45 observed at limiting substrate concentration is the result of a decrease in the Km value as opposed to the large Ca^{2+}-dependent increase of the Vmax of the native enzyme induced by calmodulin.

Marie-Paule Strub has started to characterize residues essential for the catalytic activity of bovine brain calcineurin. The primary structure of bovine calcineurin A, determined by protein sequencing, indicated that the bovine protein is composed of 80% calcineurin Aα and 20% calcineurin Aβ. Eleven of the twelve cysteines of calcineurin A are present in both isoforms. A single cysteine residue located in the putative catalytic domain was shown to be required for the p-nitrophenylphosphatase activity whereas its modification had little effect on the protein phosphatase activity of calcineurin. This observation is consistent with the different dephosphorylation mechanisms toward these two substrates exhibited by the enzyme.

A comparison of the known primary structures of calcineurin A from different organisms, including that of Drosophila melanogaster calcineurin determined in our laboratory, indicates that all the functional domains of calcineurin are highly conserved. Each of the regulatory domains is encoded by individual exons whose distribution is preserved from Drosophila to man. Thus, Ca^{2+} regulation of calcineurin appears to be important throughout evolution.

C. Physiological Roles of Calcineurin in Cellular Signalling

The selective inhibition of calcineurin by the immunosuppressant/immunophilin complex was demonstrated last year by Dr. Schreiber and his colleagues. In collaboration with Drs. Fruman, Bierer and Burakoff, we confirmed and extended this observation by demonstrating the inhibition of calcineurin by immunosuppressants in vivo. The correlation between the immunosuppressant activities of different immunosuppressant drugs and their ability to inhibit

calcineurin, demonstrated in collaboration with Dr. Schreiber's group, firmly established that a calcineurin-mediated Ca^{2+}-dependent dephosphorylation is an essential step in early events leading to T cell activation. The inhibition of calcineurin by immunosuppressant/immunophilin complexes was shown to be specific for calcineurin, thereby providing long awaited specific and potent inhibitors of calcineurin which are needed to identify additional roles for this enzyme in Ca^{2+}-mediated regulation of cellular processes.

Publications:

Stemmer P, Klee CB. Serine/threonine phosphatases in the nervous system, Current Opinion Neurobiol 1991;1:53-64.

Mackall J, Klee CB. Calcium-induced sensitization of the central helix of calmodulin to proteolysis, Biochemistry 1991;30:7242-7.

Ikura M, Spera S, Barbato G, Kay LE, Krinks MH, Bax A. Side-chain ^{1}H and ^{13}C resonance assignments and secondary structure of calmodulin in solution determined by heteronuclear multi-dimensional NMR spectroscopy, Biochemistry, 1991;30:9216-28.

Klee CB. Concerted regulation of protein phosphorylation and dephosphorylation by calmodulin, Neurochem Res 1991;16:1059-65.

Hubbard MJ, Klee CB. Exogenous kinases and phosphatases as probes of intracellular modulation. In: Chad J, Wheal H, eds. Molecular neurobiology: a practical approach. Oxford: IRL Press, 1991;135-54.

Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB, Bax A. Solution structure of a calmodulin-target peptide complex by multidimensional NMR, Science 1992;256:632-8.

Maune JF, Klee CB, Beckingham K. Ca^{2+} binding and conformational change in two series of point mutations to the individual Ca^{2+}-binding sites of calmodulin, J Biol Chem 1992;267:5286-95.

Fruman DA, Klee CB, Bierer BE, Burakoff SJ. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A, Proc Natl Acad Sci USA 1992;89:3686-90.

Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB, Schreiber SL. Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity, Biochemistry 1992;31:3896-901.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05244-15 LB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Transposable Elements in the Human Genome

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

M.F. Singer	Scientist Emeritus	LB	NCI
R.E. Thayer	Chemist	LB	NCI
G. Swergold	Senior Staff Fellow	LB	NCI
J. McMillan	Visiting Fellow	LB	NCI
V. Krek	Staff Fellow	LB	NCI

COOPERATING UNITS *(if any)* Thomas Fanning, Armed Forces Institute of Pathology; Susan Holmes, Haig Kazazian, Department of Pediatric Genetics, The Johns Hopkins University; K. Ozato and K. Becker, Laboratory of Developmental & Molecular Immunity, NICHHD

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Gene Structure and Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.50	4.25	0.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Transcription and translation of L1Hs elements is most abundant in human teratocarcinoma and epithelial-derived tumor cell lines. Our work is aimed at elucidating the mechanism and regulation of L1Hs transcription and translation as a basis for understanding L1Hs transposition in the human genome.

We have dissected further the array of DNA segments in the L1Hs 5' untranslated region that contribute to the regulation of a downstream reporter gene. Insertion of 4 extra base pairs after residue 98 decreases gene expression more than 10-fold. The region from bp 385 to 525 enhances gene expression about 10-fold in a manner independent of either orientation or location. We have begun identifying proteins that bind to sequences in the 5' UTR as potential regulators of transcription. At least four such proteins and their binding sites have been recognized between bp 1 and 526. Two are known transcription factors: UCRBP (bp 9-21) and SP-1 (bp 426-452). The two others recognize segments 80-85 and 504-526.

The difference in mobility in SDS-PAGE between the p40 polypeptides encoded by the 338 amino acid long ORFls of two different L1Hs elements, L1.2A and cD11, is associated with four amino acid differences in the central region of p40. The L1.2A p40 has the same mobility as the endogenous p40 in teratocarcinoma cells; this finding adds to accumulating evidence indicating that L1.2A and its alleles at the LRE-1 locus on human chromosome 22 are active mobile elements. The central region of p40 includes a segment which can, in principle, form a leucine zipper in either a homo- or heterodimeric coiled-coil. Evidence that p40 in teratocarcinoma cells is in multimeric complexes has been obtained by cross-linking experiments.

The endogenous p40 continues to be synthesized after differentiation of NTera2D1 cells with retinoic acid.

Project Description

Objectives:

Human LINE-1 elements (L1Hs) and similar class II (or nonLTR) retrotransposons in a wide variety of eukaryotes, are characterized by an absence of long terminal repeats, an A-rich stretch at the 3' end of the strand that carries long open reading frames, and coding sequences that predict a reverse transcriptase. The overall 5' to 3' structure of an L1Hs genomic consensus sequence and of cDNAs derived from cytoplasmic, polyA$^+$ RNA of human teratocarcinoma cells is as follows: a 900 bp 5' untranslated region, an open reading frame ORF1 encoding a 338 amino acid polypeptide called p40, an interORF region of about 33 bp bounded by stop codons, a 3852 bp ORF2 that includes a region encoding a polypeptide homologous to known reverse transcriptases, a 200 bp 3' untranslated region, and an A-rich stretch of variable length.

Our work on the transcription and translation of L1Hs benefited greatly during the last year by the demonstration by Haig Kazazian and his colleagues at The Johns Hopkins University, that alleles at the LRE-1 locus on human chromosome 22 likely encode transpositionally active L1Hs elements. One such allele, L1.2A, was cloned and sequenced by the Hopkins group and has been available to us. A second allele, L1.2B, was also sequenced. The L1.2A ORF2 was shown by the Hopkins group to encode an active reverse transcriptase when appropriately introduced into yeast cells. L1.2A and L1.2B differ in only three base pairs out of the 6 kbp element and these are in ORF2. It was concluded, on the basis of sequence similarity, that L1.2B was the source of a newly transposed L1Hs detected earlier in the factor VIII gene of a patient with hemophilia A.

Our aim is to understand the mechanism and the regulation of L1Hs transcription and translation in order to elucidate the mechanism and control of transposition. Previous work demonstrated that among all cell lines tested, L1Hs expression is most abundant in human teratocarcinoma cell lines and in some tumor cell lines of epithelial origin. This conclusion is based on one or more of the following criteria: 1) detection of cytoplasmic, polyA$^+$ L1Hs transcripts, 2) detection of p40 using specific rabbit antisera elicited with an *E. coli* synthesized trpE-ORF1 fusion protein, and 3) the specificity of the 5' UTR in supporting expression of a reporter gene upon transfection. All three criteria are met for the human teratocarcinoma lines tested.

Major Findings:

A. L1Hs Transcription

Properties of p40. The p40 in 2102EP and NTera2D1 human teratocarcinoma cells is mainly if not entirely cytoplasmic as evidenced by immunofluorescence on whole cells and cell fractionation studies followed by Western blotting. If Mg^{2+} is present in the extraction buffer, the p40 in the cytoplasmic fraction is distributed in both the pellet and supernatant fractions after centrifugation at 12,000xg for 10 minutes. If the Mg^{2+} is removed by dialysis against EDTA prior to centrifugation, the majority of the p40 remains in the supernatant fluid after such centrifugation. In the absence of Mg^{2+}, p40 in the cytoplasmic extracts partitions into two fractions on a Sephacryl S-400 column; one elutes

between markers of 100 and 200 kDa and the other with a 44 kDa marker, as expected for a p40 monomer.

These observations have been somewhat clarified by cross-linking experiments which indicate that p40 may be complexed with itself or other polypeptides in teratocarcinoma cell cytoplasms. Thus, Western blots of SDS-PAGE gels indicate that after treatment for 10 minutes with 0.005 percent glutaraldehyde, the endogenous p40 is found in three size classes; the monomer band with a mobility equivalent to a polypeptide of 38 kDa and two groups of bands with mobilities approximately corresponding to 80 and 160 kDa, respectively. In the absence of glutaraldehyde treatment, all the p40 is in the band at 38 kDa. If the glutaraldehyde concentration is raised to 0.02 percent, only the group of bands corresponding to 160 kDa is observed.

The ability of p40 to form multimeric complexes, either with itself or with other polypeptides, may be conferred by the putative leucine zipper predicted by the amino acid sequence between residues 90 and 131. The presence of this domain was noted in last year's report. Additional analysis of the L1.2A p40 suggests six possible heptad repeats, four with leucines and two with methionines at position "d" of the successive helical turns. In position "a", which is generally occupied by hydrophobic residues in coiled coils, four out of six of the p40 heptads conform. The other two heptads have cysteine residues at position "a" and modeling and experimental work (by others) suggests that these could form disulfide bridges between two interacting coils. Positions c, e, and g are predominantly charged in the p40 putative leucine zipper, and these could stabilize a coiled coil through intra- and interhelical polar interactions, as has been demonstrated for structures such as the GCN4 leucine zipper.

Treatment of cytoplasmic extracts of 2102EP cells with both alkaline phosphatase and potato acid phosphatase causes an increase in the mobility of the endogenous p40 on SDS-PAGE. Also, when NTera2D1 cells were incubated with ^{32}P-labeled orthophosphate, extracted, and the p40 immunoprecipitated with the specific antiserum and analyzed by SDS-PAGE, the p40 band was radiolabeled. These experiments confirm the preliminary conclusion reported last year that p40 is phosphorylated in these cells.

Expression of p40 in NTera2D1 cells after treatment with retinoic acid. Some years ago, we reported the detection of full length, cytoplasmic, polyA$^+$ L1Hs transcripts in NTera2D1 cells and the disappearance of that RNA upon differentiation of the cells with retinoic acid. We have now investigated the occurrence of p40 under similar conditions. The p40 polypeptide, migrating as if it were 38 kDa on SDS-PAGE, is detectable in NTera2D1 cells after several weeks of retinoic acid treatment; visual inspection of the cells indicated that they had differentiated. We then investigated whether p40 was actually being synthesized by the differentiating cells. The synthesis of p40 was monitored by pulse labeling cells with ^{35}S-methionine at various times up to 18 days after initiation of retinoic acid treatment. The polypeptide was immunoprecipitated and the precipitated material analyzed by SDS-PAGE. The cells continued to synthesize p40 at all times tested, and at approximately the same rate as before retinoic acid treatment began. Differentiation was monitored with monoclonal antibody to a cell surface marker A_2B_5, showing that greater than 95 percent of the cells were differentiated after 7 days treatment with retinoic acid.

The p40 encoded by L1.2A. One possible explanation for the discrepancy between the disappearance of the L1Hs cytoplasmic RNA and the continued synthesis of p40 is that only a small portion of the RNA is active mRNA for p40 synthesis. This explanation could also resolve another curious observation. As reported last year, the p40 expressed by the ORF1 of L1.2A has the same electrophoretic mobility in SDS-PAGE as does the endogenous p40 in human teratocarcinoma cells. This is in contrast to several L1Hs elements isolated from cDNAs prepared from the L1Hs cytoplasmic RNA. One cDNA in particular, cD11, yields a p40 that migrates more slowly than endogenous p40, when produced after transfection of NTera2D1 cells with cD11. Yet, cD11 and L1.2A encode p40s of identical length. Constructs in which pieces of the ORF1 of L1.2A were replaced by their cognate fragments from cD11 were prepared and the electrophoretic mobilities of the resultant p40s were analyzed by Western blotting of extracts of transfected NTera2D1 cells. The amino acid sequence of the central third of the cD11 protein accounted for most of its characteristic mobility. This region contains the leucine zipper. Thus, it appears that the endogenous p40 must be expressed from only a limited subset of L1Hs cytoplasmic RNAs in teratocarcinoma cells. These RNAs could persist after retinoic acid treatment but be too rare to be detected by Northern blotting; recently the RNA has been detected by PCR. These data also indicate that L1.2A could give rise to the observed endogenous p40, consistent with its identification as an active transposable element.

Translation of ORF2. We have produced a number of high titer rabbit antisera to various regions of the polypeptide predicted by ORF2. These antisera immunoprecipitate *in vitro* translation products of ORF2 and at least one recognizes the TyA-ORF2 fusion polypeptide shown (by Kazazian and coworkers) to have reverse transcriptase activity.

B. L1Hs Transcription

Regulation of L1Hs Transcription. During this year we concluded a series of experiments designed to identify regions of the L1Hs 5' UTR that are capable of acting as transcriptional regulators for a reporter gene. The critical nature of the region around bp 100 was further highlighted by finding that insertion of only 4 bp at this location causes a 13-fold reduction in gene expression. The region of the 5' UTR previously shown to be required for high level L1Hs mRNA accumulation (bp 1-663) was cloned in 4 fragments into an enhancerless SV40 promoter driven CAT gene construct. The fragment between bp 385 and 525 enhanced the expression of CAT about 10-fold. This activity was orientation and location independent and was seen only in NTera2D1 cells but not in HeLa or CV-1 cells, suggesting that it is specific for cell type.

Efforts to identify proteins that may bind to the regulatory regions in the 5' UTR in a sequence specific manner have been initiated. Conditions were devised to permit the growth of NTera2D1 cells in spinner culture and unfractionated nuclear extracts were prepared. Three regions of the 5' UTR have been studied thus far. Band shift assays reveal one or more complexes with bp 1-40; in collaboration with Keiko Ozato's group, we demonstrated that the ubiquitous transcription factor UCRBP (also called YY1, NF-E1, and δ) also shifts bp 1-40 and gives complexes of the same mobility as the NTera2D1 cell extracts. The region 1-40 contains a single copy of a typical UCRBP binding site. The region from bp 80 to 120 is shifted differently by extracts from NTera2D1 and 2102EP

teratocarcinoma cells compared to HeLa, Jurkat, and Raji cells. Competition with any oligonucleotide that contains bp 80-85 competes for the teratocarcinoma cell-specific binding but randomized oligonucleotides or those from other regions of the 5' UTR do not. Preliminary experiments indicate that a nuclear protein may bind to the region bp 504-526. DNAse I footprinting experiments indicate that purified transcription factor SP-1 binds between bp 426 and 452. Thus, four different protein binding sites have been identified of which two interact with known transcription factors, UCRBP and SP-1. The nature of the other two proteins is unknown.

Publications:

Goldfarb LG, Brown P, McCombie WR, Goldgaber D, Swergold GD, Wills PR, Cerenakova L, Baron H, Gibbs Jr, CJ, Gajdusek DC. Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene, Proc Natl Acad Sci USA 1991;88:10926-30.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05258-13 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

Molecular Studies of Eukaryotic Gene Regulation

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

B.M. Paterson	Chief, Biochemistry of Gene Expression Section	LB	NCI
J. Eldridge	Biochemist	LB	NCI
Q. Wei	Visiting Fellow	LB	NCI
M. Shirakata	Visiting Fellow	LB	NCI
I. Karsch-Mizrachi	Visiting Scientist	LB	NCI
D. Hursh	Staff Fellow	LB	NCI
K. Mitsui	Visiting Associate	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Biochemistry of Gene Expression Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.50	7	0.50

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have continued our studies on the genes involved in cellular commitment and differentiation during myogenesis. In addition to the expression of the structural genes characteristic of differentiated muscle, myogenesis involves the interaction of four myogenic determination genes in the vertebrates, MyoD, myogenin, Myf 5, and MRF4, the MyoD family of myogenic regulators. These genes are thought to play a role in muscle cell commitment in the somites as well as the transcriptional regulation of the muscle structural genes during differentiation. We have isolated all four of these genes from the chicken and have focused most of our attention on the avian MyoD homolog, CMD1, since it is expressed in the somites, the myoblast, and in the newly differentiated muscle fiber. These myogenic regulatory factors are phosphorylated and bind to their target sequences as dimers. We have analyzed the role of phosphorylation and the nature of the dimer (either homo or heterodimer) in CMD1 function. In the latter case, this has led to the characterization of at least two and possibly three proteins involved in heterodimer formation in myoblasts and fibers that may modulate CMD1 function. In order to take a genetic approach to the study of myogenesis, we have isolated the MyoD homolog from Drosophila, called Dmyd or nau. Dmyd is the only MyoD homolog in Drosophila, it is transiently expressed in a subset of muscle precursor cells in a segment specific pattern, and the majority of larval muscles contain at least one Dmyd positive cell. By studying the upstream and downstream genes in the myogenic pathway in Drosophila we hope to identify correlates in the vertebrates.

Objectives:

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

Major findings:

A. Isolation and Characterization of the Four Avian Myogenic Regulatory Factors.

The four avian myogenic regulatory factor cDNA clones have been isolated by virtue of their weak cross hybridization under low stringency conditions to the mouse MyoD or the human Myf4 cDNA clones. Avian MyoD (CMD1) and myogenin (CMgn) were isolated from a lambda gt10 library. The avian Myf5 (CMyf5) and MRF4 (CMRF4) clones were initially isolated from a genomic library. Specific genomic fragments were then used to isolate the cDNA clones. CMD1 and CMgn have been characterized in the most detail. CMD1 encodes a protein smaller than MyoD1, 298 versus 318 amino acids, respectively, and is 80% concordant. The DNA binding (basic region) and dimerization domains (helix-loop-helix region) are completely conserved except for a serine (mouse) to threonine (avian) change in the loop. CMgn is significantly smaller than myogenin, 226 versus 287 amino acids, respectively. Within the 61 amino acids of the basic helix-loop-helix domain there are 12 amino acid changes compared to CMD1 and 3 changes compared to myogenin. Like CMD1, CMgn will convert 10T1/2 mouse fibroblasts to muscle, bind to the MCK enhancer as a heterodimer with the E2a protein E12, and activate muscle specific promoters in a cotransfection assay. Interestingly, over expression of CMgn in 10T1/2 cells does not cause withdrawal of the cells from the cell cycle as does over expression of CMD1. The basis for this difference is being investigated.

In mammals, Myf5 and MRF4 are linked and the genomic library screen indicates the avian genes are also linked by a short spacer region that may contain promoters for both genes since they are transcribed in opposite directions away from the spacer. Myf-5 and MRF4 are the first and the last myogenic regulatory factor genes, respectively, expressed in murine development. We hope to carry out similar expression studies on the avian MyoD family during chick development since our collaboration with Gary Lyons and Margaret Buckingham of the Institute Pasteur has shown the patterns for MyoD and myogenin are different in mouse and chick during somite development.

Growth of muscle cells in the thymidine analog, BUdR, reversibly inhibits myogenesis as well as the synthesis of CMD1. This is also reflected in the inhibition of muscle specific promoter activation in transfections with BUdR treated muscle cells. This block can be rescued by cotransfection with a CMD1 expressing plasmid. Expression of CMD1 in mouse 10t1/2 fibroblasts activates the endogenous MyoD gene, suggesting possible auto activation in the maintenance of the muscle phenotype which is interrupted by BUdR. Studies with the CMD1 promoter from -8Kb have not demonstrated auto activation although the promoter is muscle specific. However, transfections of 10T1/2 cells with the entire gene containing from -1Kb to -8Kb of promoter result in myogenic conversion. Cotransfections with CMD1 and human Myf 5 , the earliest factor expressed in mammalian somites, does not rescue CMD1 activation of the CMD1 promoter. Deletion studies on the promoter indicate it is muscle specific and non functional in BUdR treated cells with deletions to -169 but no deletion will respond to trans CMD1. We assume activation of the CMD1 promoter requires an additional factor(s) present in muscle cells.

B. Expression of the Myogenic Regulatory Factors in Baculovirus

In an effort to study the biochemical properties of the myogenic regulatory factors, we have developed a novel system for the expression of these proteins in Baculovirus infected sf9 cells. This eukaryotic expression system gives reasonable yields of appropriately modified proteins, phosphorylated or

glycosylated, that more closely resembles the protein expressed in muscle cells. We have introduced two modifications that make isolation of the recombinant virus and the expressed protein much easier than with the standard procedures. We have modified the expression vector so that the recombinant virus contains the protein of interest under the control of the strong polyhedron promoter and the human IL-2 gene under the control of a weaker early viral promoter in the opposite orientation. Cells containing the recombinant virus can be isolated through the expression of the IL-2 receptor. We have coupled IL-2 MAb to magnetic beads and this reagent is used to sort the infected cells of interest. Secondly, we have introduced a histidine tag at the amino terminus of the proteins to facilitate isolation on Ni-agarose columns. The histidine tagged CMD1 expressed under a viral LTR behaves identically to unmodified CMD1 in all our assays. The initial procedure has been worked out with CMD1 and has recently been extended to all the myogenic regulatory factors under study in the lab.

The baculo expressed CMD1 is a phosphoprotein with a phosphopeptide pattern and phosphoamino acid composition indistinguishable from authentic CMD1 affinity purified from primary muscle cultures. When this protein is injected into cells it is nuclear in location. We are testing its ability to activate a LacZ reporter construct in collaboration with Dr. Eric Olson at M.D. Anderson in Houston, Texas. The His-baculoCMD1 will dimerize with the E2a gene product ,E12, made either in retic lysates or purified from E. coli and will bind specifically to the E-box in the MCK enhancer in gel shift assays, as determined with mutant oligos and DNA foot print assays. The most interesting result indicates the phosphorylation can regulate homodimer formation and reduce heterodimer binding to the E-box approximately three fold. We are in the process of mapping the position of the phosphoaminoacids and hope to identify kinase activities that will block homodimer formation and restore heterodimerization to full levels. A specific cyclic AMP kinase site may be a potential regulatory region. This kinase site will be mutated to see if phosphorylation is important in the homo-heterodimer interaction and if CMD1 is still functional as an activator. We intend to study the role of phosphorylation in the regulation of CMD1 function in more detail.

C. Structural Analysis of CMD1

Efforts to produce the basic helix-loop-helix domain of CMD1 in sufficient quantities for NMR studies has been difficult. Although we can make small amounts of the protein in E.coli with the T7 system, yields are too low for NMR analysis. We have tried to improve yields by using E. coli strains that block low levels of T7 expression in the absence of IPTG but this has proven to be of limited success. In addition, we have constructed a mini gene using optimal E. Coli codons. If this fails, this approach will be discontinued.

D. Isolation of the Avian E Proteins

Using the mammalian sequences for the E2a gene proteins, E12 and E47, we have designed PCR primers to generate probes to screen avian genomic and cDNA libraries for the homologs to these mammalian proteins. This has led to the isolation of a full-sized E12 homolog and two different, partial E47 homologs. These proteins contain the basic helix-loop-helix domain necessary for dimerization and DNA binding. All three E-protein homologs heterodimerize with CMD1 and bind to the MCK E-box in gel shift assays. RNA for the avian E proteins is more abundant in muscle as compared to liver or brain. A detailed study of E12/E47 expression during development and myogenesis is under way using specific PCR primers to determine if there is a preferential pattern of expression. We are trying to determine if these proteins are indeed the in vivo partners for CMD1 or if other related proteins are the dimer partners. Preliminary results from immunoprecipitation assays under low and high stringency with antibodies to CMD1 and the E2a proteins suggest CMD1 is dimerized with these avian E2a proteins in primary muscle cultures. This is also being analyzed in COS cells where higher levels of protein can be produced. We are measuring the ability of the E-

proteins to activate an E-box reporter construct [(E5-E2)4CAT from T. Kadesch] with and without CMD1 in various cell backgrounds in order to study activation and partner interactions.

E. The Isolation and Characterization of the Drosophila Homolog to the Vertebrate Myogenic Regulatory Factors.

We have isolated a cDNA clone, called Dmyd for Drosophila myogenic-determination gene, that encodes a protein with structural and functional characteristics similar to the members of the vertebrate MyoD family of proteins. The Dmyd clone encodes a polypeptide of 332 amino acids with 82% identity to MyoD in the 41 amino acids of the putative helix-loop-helix region and 100% identity in the 13 amino acids of the basic domain proposed to contain the essential recognition code for muscle-specific gene activation. Low stringency hybridizations and PCR analysis with degenerate primers indicate Dmyd is not a member of a multigene family similar to MyoD in the vertebrates. Dmyd is a nuclear protein in Drosophila, consistent with its role as a nuclear-gene regulatory factor, and is proposed to be a transiently expressed marker for muscle founder cells. We have used an 8Kb promoter fragment from the gene, which contains the first 55 amino acids of Dmyd, joined to lacZ, to follow myogenic precursor cells into muscle fibers with antibodies to beta galactosidase and to Dmyd. Unlike the myogenic factors in vertebrate muscle cells, Dmyd appears to be expressed at a much lower level in differentiated Drosophila muscles, so Dmyd cannot be followed continuously as a muscle marker. This fact is reflected in the loss of Dmyd RNA expression in 12 to 24 hour embryos, a major period of early myogenesis, as well as in the undetectable level of the nuclear antigen in primary cultures of embryonic and adult Drosophila muscle.

P element mutagenesis experiments have been unsuccessful to date. We have several P element and Hobo insertions near the gene at 95 A/B and intend to generate deficiencies through jumping and gamma irradiation. This will be carried out in conjunction with a classical EMS mutant screen. Preliminary studies with ectopic expression of Dmyd under the heat-shock promoter have given no phenotype in later development but this must be explored in greater detail. We are in the process of generating more Dmyd promoter lacZ lines by jumping the P element to make sure the pattern of lacZ expression is correct. These lines will be crossed with the heat-shock lines to study auto activation. We have received a cold sensitive Ricin A chain mutant from Cahir O'Kane (Warwick University, London) in a P element construct to express Ricin A toxin under the control of the Dmyd promoter in order to knock out the putative Dmyd muscle founder cells at different stages of development. This approach has been successfully used to study eye development in Drosophila. It is not clear what the target genes for Dmyd are since muscle specific genes are active in the absence of the Dmyd gene when using a large deficiency (S. Abemyer). Dmyd, unlike the vertebrate factors, forms homodimers that bind to certain E-box consensus sequences. We would like to look for possible Dmyd partner proteins and define the target genes. The LacZ construct is being crossed to various heat shock lines expressing well known regulators of mesoderm formation, such as Ubx and dpp, in a effort to look for genes involved in the activation of Dmyd. The initial part of this work was carried out in Basel, Switzerland, while Dr. Paterson was on sabbatical with Dr. Walter Gehring in order to develop a genetic approach to the study of myogenesis.

F. Studies on the Structure of the Myogenic Regulatory Factors

Our preliminary studies with the expression of Dmyd in mouse 10T1/2 fibroblasts indicated that although the amino acid sequence of basic helix-loop-helix (HLH) region in Dmyd was 85% identical to the vertebrate factors and the hydrophobic residues were conserved exactly, Dmyd did not convert the fibroblasts to muscle as the vertebrate factors did. Dmyd was expressed at high levels and was a good nuclear antigen in mouse fibroblasts as judged by antibody staining. It was previously proposed that the highly conserved hydrophobic amino acids in the HLH region mediate dimerization. We have analyzed the amino acid sequence requirements for dimerization between the E2a gene product, E12,

and CMD1 using chimeric mutants of the HLH domains. We demonstrate that specific polar and charged amino acid residues in the amphipathic helices of the CMD1 HLH domain are required for the selective dimerization with E12. A back mutation in E12 restores normal levels of dimerization with a poorly dimerizing CMD1 mutant, suggesting the interaction of particular hydrophilic side chains in the dimer. A parallel four-helix bundle model for the structure of the HLH dimer is proposed to explain these results. Various mutants in the HLH region are under investigation to determine those residues critical to specifying the dimer interactions. We hope this will tie in with our phosphorylation studies.

References

Paterson BM, Walldorf U, Eldridge J, Dubendorfer A, Frasch M, Gehring WJ. The Drosophila homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells, Proc Natl Acad Sci USA 1991;88:3782-6.

Lyons GE, Muhlebach S, Moser A, Masood R, Paterson BM, Buckingham ME, Perriard J-C. Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle, Development 1991;113:1017-29.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05262-12 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Eukaryotic Gene Regulation and Function: The Metallothionein System

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

D. H. Hamer	Chief, Gene Structure and Regulation Section	LB	NCI
W. Yang	Visiting Fellow	LB	NCI
F. Otsuka	Visiting Fellow	LB	NCI
M. Takao	Visiting Fellow	LB	NCI
R. Sharon-Frilling	Visiting Fellow	LB	NCI
S. Hu	Chemist	LB	NCI
A. Dobi	Visiting Fellow	LB	NCI

COOPERATING UNITS *(if any)*
Mike Summers and Jose Casas-Finet, U. of Maryland at Baltimore; Dennis Winge, U. of Utah

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Gene Structure and Regulation Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.00	6.00	-

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

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

Metal ions can both activate and repress eukaryotic gene expression. In yeast cells, Cu activates metallothionein gene expression by directly interacting with the DNA-binding domain of the ACE1 transcription factor. We have obtained evidence that the 11 critical cysteine residues of ACE1 are organized into two subclusters that interact with distinct regions of the upstream activation sequence. Cu also represses the activity of several genes involved in Cu uptake and utilization, including the cell surface reductase gene FRE1. We have identified and cloned a new regulatory factor, ACU1, that is involved in this repression pathway. ACU1 is weakly homologous to ACE1, and contains a cysteine-rich motif characteristic of Cu-binding proteins. Thus, each of the proteins that we have shown to be critical for Cu homeostasis in yeast appears to bear a common structural element, which we have dubbed the "Cu fist".

Projection Description

Objectives:

To understand how eukaryotic cells regulate gene expression in response to changes in the cellular environment.

Major Findings:

A. ACE1

When yeast cells are exposed to a high concentration of Cu, they respond by activating the expression of the CUP1 gene, which encodes a Cu-binding metallothionein. Transcriptional induction is mediated by the ACE1 transcription factor, which consists of two domains: an amino-terminal, Cu-dependent DNA-binding domain, and a carboxy-terminal, acidic activation domain. We previously reported genetic and biophysical evidence that Cu directly interacts with 11 critical cysteine residues within the amino-terminal domain of ACE to form a polynuclear Cu(I)-S cluster that organizes the protein for DNA recognition.

To further probe the structure and organization of this unique DNA-binding domain, we performed both cysteine modification and detailed DNA interaction studies. Cysteine reactivity was probed by carboxymethylation of ACE1 in the presence of 0, 4 or 7 equivalents of Cu(I). DNA interactions were studied by gel mobility shift experiments with a series of 42 truncated or mutated oligonucleotides, and by missing contact, methylation interference and ethylation interference footprints. The results show that 1) Cu binds to ACE1 in a stepwise cooperative fashion, probably forming two subclusters; 2) cysteine residues at positions 11, 14, 23 and 80 are in the second, apparently weaker subcluster; and 3) the stronger subcluster preferentially binds to the 3' portion of the recognition sequence whereas the weaker subcluster occupies the 5' sequences. Based on these data, we propose a two subcluster model for the DNA-binding domain of ACE1.

B. ACU1

When yeast cells are exposed to low levels of Cu, they respond by repressing the expression of the FRE1 gene, which encodes a Fe(III)/Cu(II)→Fe(II)/Cu(I) reductase, and other genes involved in Cu uptake. We isolated and cloned the gene corresponding to a dominant mutant, ACU1-31 (previously referred to as upc31), in which reductase and uptake activities are constitutively derepressed. The phenotype of null mutants indicates that ACU1 is a Cu-sensitive activator of the reductase and uptake systems.

ACU1 encodes a nuclear protein, as demonstrated by immunoflourescence experiments using β-galactosidase fusion proteins, and the amino-terminal 40 amino acids are 30% homologous to the corresponding region of ACE1. In addition, there is a repeated cysteine-rich motif characteristic of Cu and Fe-binding proteins. Studies on the interaction between metal ions, ACU1 and DNA are in progress.

C. Mammalian Factor

Due to the lack of good genetic selections, progress on metal-dependent regulatory factors from mammalian cells has been slower than for yeast. The putative metallothionein regulatory factor previously termed MBF1 was cloned and shown to be identical to the DNA replication accessory protein RAP1. Another putative factor detected by a yeast complimentation strategy was cloned and shown to encode a short peptide with a potential Zn finger motif, but DNA-binding activity has not been detected. ZAP1, a Zn-regulated binding factor, is still being purified.

D. Termination Notice

The original objective of this project was to understand how cells induce metallothionein gene transcription in response to metal ions. This objective has been fully met in the yeast cell system. Therefore, the project will be terminated in the coming year, and the focus of the group will turn to sexual differentiation as explained in an accompanying Progress Report.

Publications:

Dameron CT, Winge DR, George GN, Sansone M, Hu S, Hamer DH. A copper-thiolate polynuclear cluster in the ACE1 transcription factor, Proc Natl Acad Sci USA 1991;88:6127-31.

Yang W, Gahl W, Hamer DH. Role of heat shock transcription factor in yeast metallothionein gene expression, Mol Cell Biol 1991;11:3676-81.

Casas-Finet JR, Hu S, Hamer D, Karpel RL. Spectroscopic characterization of the copper(I)-thiolate cluster in the DNA-binding domain of yeast ACE1 transcription factor, FEBS Lett 1991;281:205-8.

Hamer DH. Essential and toxic heavy metal metabolism: the role of metallothionein. In: Trace elements in nutrition, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05263-11 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Eukaryotic Chromatin Structure and Gene Regulation

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

C. Wu	Chief, Developmental Biochemistry Section	LB	NCI
J. L. Brown	Visiting Associate	LB	NCI
S. Rabindran	Staff Fellow	LB	NCI
G. Lavorgna	Visiting Associate	LB	NCI
J. Clos	Visiting Fellow, departed 1/92	LB	NCI
J. Westwood	Guest Researcher, departed 3/92	LB	NCI
J. Wisniewski	Visiting Fellow	LB	NCI
R. Haroun	Hughes Medical Student		
Michael Kim	Visiting Fellow	LB	NCI
T. Tsukiyama	Visiting Fellow	LB	NCI
B. Davis	Laboratory Worker	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Developmental Biochemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.25	7.75	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This group has continued its study of three sequence-specific DNA-binding proteins in Drosophila, the heat shock transcription factor HSF, and two factors which regulate the segmentation gene fushi tarazu, FTZ-F1 and tramtrack. With the cloning of the HSF gene from human in the previous year, a comparative study of HSF function in human and Drosophila is under way. Considerable progress has been made in the molecular genetic analysis of the regions of the HSF protein that are important for its regulation. The most interesting result regarding HSF regulation has been the demonstration of the importance of the C-terminal leucine zipper motif in maintaining the inactive HSF conformation. Another significant achievement was the successful demonstration of the proposed function of the tramtrack protein as a repressor of the ftz gene. An additional highlight came from the immunostaining analysis of FTZ-F1 on Drosophila polytene chromosomes which revealed a new function for FTZ-F1 as an intermediate regulator of genes expressed at the onset of metamorphosis. These findings further affirm the phenomenon of regulatory pleiotropy, and suggest that many transcription factors regulating developmental genes are utilized repeatedly throughout the life of the organism to serve different regulatory pathways.

GPO 914-918

Project Description

Objectives:

I. Induction of Heat Shock Genes

A. Structure and Regulation of Drosophila and Human HSF Proteins

1. (Tim Westwood, departed 3/92). The native state of Drosophila HSF has been analyzed by glycerol gradient centrifugation and gel exclusion chromatography. The HSF protein behaves as an monomer in the inactive state and as a trimer when activated. Both forms of HSF protein appear to be highly asymmetric, which provides an explanation for the aberrant migration of HSF as an apparent dimer and hexamer on nondenaturing polyacrylamide gels. On the basis of the available evidence, the heat shocked induced aggregation of HSF appears to be caused by a change in the aggregation state from monomer to trimer. The trimeric state, with three DNA-binding domains held in proximity by leucine zipper-type interactions, binds the heat shock element, a three-fold alternating repeat of nGAAn, with high affinity.

2. (Michael Kim, arrived 4/92). Biophysical studies are under way to analyze the structure of the DNA binding and oligomerization domins of HSF. A recombinant DNA construct carrying a 130 amino acid region of HSF comprising the DNA binding domain has been over-expressed in E. coli, and soluble protein has been purified by chromatography to over 95% homogeneity and concentrated to the 10 mg/ml range. This domain will be employed for studies on the analytical ultracentrifuge and for NMR analysis in collaboration with Dr. Ad Bax.

3. (Joachim Clos, departed 1/92;Jan Wisniewski, arrived 11/91). The regulation of Drosophila and human HSF proteins has been investigated by cross-species expression of the human protein in Drosophila cells and vice versa, by means of transient transfection assays. Interestingly, the human HSF protein assumes the induction properties of Drosophila HSF when expressed in Drosophila cells. In particular, the induction temperature for human HSF monomer to trimer transition was reset from 44 to 37 degress C. In human cells, Drosophila HSF was found to be induced at low and high temperatures. These results indicate that the regulation of HSF oligomerization is not simply dependent in the primary structure of the protein itself. It is possible that secondary modifications of HSF that are specific to human and Drosophila cells are responsible for its regulation, or that other components (e.g., ionic differences or molecular chaperones) are necessary for proper regulation.

4. (Sridhar Rabindran/Raymond Haroun). A sequence comparison between human and Drosophila HSF cDNAs previously revealed a conserved C-terminal leucine zipper motif, which was proposed to have a regulatory function in HSF oligomerization. During this year, this hypothesis was tested by constructing point mutations in the zipper motif. When transfected in homologous cells, these mutations were found to cause a partial increase in aggregation and DNA binding activity of human HSF at the nonshock temperature. In addition, deletion mutants which remove C-terminal sequences separate from the zipper motif also cause constitutive activation of human HSF. Together these results indicate that both C-terminal sequences and the zipper motif participate in regulating the assembly

of the inactive, monomeric state of HSF protein. Deletion analyses performed on the Drosophila HSF molecule are consistent with the data obtained with human HSF.

5. (Sridhar Rabindran/Raymond Haroun). Interactions of HSF with hsp70 were investigated by co-immunoprecipitation experiments. It was found that the major protein that showed interaction with HSF by this assay was the heat shock protein hsp70. This interaction was observed under both normal and heat shock conditions, and may not be directly related to the regulation of HSF. Preliminary mapping of the site of interaction using the human HSF deletion mutants suggests the importance of the C-terminal region.

6. (Gisele Giorgi). The genomic structure of human HSF1 was determined by the isolation of a series of overlapping phage clones that contained human HSF1 cDNA sequences. Restriction fragments carrying cDNA sequences were subcloned and sequenced. The sequence analysis revealed the presence of 13 exons for human HSF1. By contrast, the Drosophila HSF ORF contained 7 exons. For both human and Drosophila proteins, the DNA binding domain is encoded in three separate exons, suggesting the possibility of a modular assembly of the HSF DNA binding domain. The position of the intron-exon junctions separating the three conserved exons is identical between human and Drosophila HSFs, indicating that this arrangement of exons predates the divergence between insects and mammals over 600 million years ago. One other intron-exon junction was found to be conserved, located on the C-terminal side of the fourth leucine zipper motif.

7. (Toshio Tsukiyama). With the departure of Peter Becker in July 1991, we suspended our studies of the chromatin structure and transcriptional regulation of the hsp70 gene, until Toshio Tsukiyama's arrival in March 1992. These studies are now being continued with the fine structure analysis of transcription factor-DNA interactions at the hsp70 promoter. In particular, cooperative and competitive interactions between the three proteins known to bind to the hsp70 promoter (GAGA factor, TFIID, and HSF) will be analyzed in the context of free DNA and assembled chromatin, using the Drosophila embryo chromatin assembly system developed in the past year from this group.

II. Regulation of the fushi tarazu Gene

In previous studies from this group, two DNA binding factors that interact with the Drosophila homeobox fushi tarazu (ftz) segmentation gene were identified and cDNAs for these proteins were subsequently cloned: FTZ-F1, a new member of the steroid receptor superfamily, and FTZ-F2, now called tramtrack, a zinc finger protein.

A. FTZ-F1, a Transcriptional Activator of ftz and Intermediate Regulator in the Ecdysone-induced Genetic Cascade (Giovanni Lavorgna)

FTZ-F1 binds to a common sequence at multiple sites located in cis-regulatory and coding regions of ftz. Mutational studies of ftz-lacZ genes introduced into flies by germline transformation suggest that FTZ-F1 is a transcriptional activator of ftz. In this year's studies, a variant of FTZ-F1 (called late FTZ-F1) that has 400 residues replaced on the amino-terminal side with a different sequence was cloned and sequenced. Late FTZ-F1 is present in late embryos,

larvae and adults. A function for late FTZ-F1 was determined. During the larval to pupal transition, late FTZ-F1 was found to be activated as the product of the 75CD chromosomal puff in salivary gland polytene chromosomes. The 75CD prepupal puff is induced in response to the insect hormone ecdysone, and has been postulated to be an intermediate regulator in the genetic cascade of about 100 different loci which are found to be sequentially activated in response to ecdysone. Immunostaining of FTZ-F1 protein on polytene chromosomes and direct DNA binding studies support this hypothesis.

B. Tramtrack, a Transcriptional Repressor of the ftz (Lesley Brown)

The FTZ-F2 protein behaves as a negative regulator of ftz, since mutations in a cis-regulatory region of ftz which disrupt FTZ-F2 binding to DNA result in derepression of ftz-lacZ constructs in transformed flies. FTZ-F2 is identical to the tramtrack zinc finger protein reported by Travers, and will henceforth be called tramtrack. Immunostaining of tramtrack protein shows a complex expression pattern throughout embryogenesis. The most intriguing aspect of the studies with tramtrack relates to the timing of the ftz-lacZ derepression caused by tramtrack binding site mutants. Derepression is evident before the syncytial blastoderm stage, as early as the third nuclear division cycle, suggesting that early developmental genes such as ftz may require active repression to prevent precocious expression. In support of this model, germline transformants carrying the tramtrack gene under control of the heat shock promoter show repression of the endogenous ftz gene when heat shocked.

The studies with FTZ-F1 and tramtrack, which are both maternally and zygotically expressed, reveal a class of developmental regulators that can be utilized repeatedly throughout the life of the organism to serve different regulatory pathways. As such, their mutant phenotypes would be unspecific, and would not lead to identification of these genes in standard genetic screens. We suspect that regulatory pleiotropy is a property of a large number of transcription factors.

Publications:

Brown JL, Sonoda S, Ueda H, Scott MP, Wu C. Repression of fushi tarazu (ftz) segmentation gene expression, EMBO J 1991;10:665-74.

Becker PB, Rabindran SK, Wu C. Heat shock regulated transcription in vitro from a reconstituted chromatin template, Proc Natl Acad Sci USA 1991;88:4109-13.

Rabindran SK, Giorgi G, Clos J, Wu C. Molecular cloning and expression of a human heat shock factor, HSF1, Proc Natl Acad Sci USA 1991;88:6906-10.

Wu C, Clos JC, Westwood JT, Zimarino V, Becker PB, Wilson S. Structure and function of Drosophila heat shock factor. In: Maresca B, Lindquist S, eds. Heat shock. Heidelberg: Springer-Verlag, 1991;9-16.

Lavorgna G, Ueda H, Clos J, Wu C. FTZ-F1, a new hormone receptor homolog implicated in the activation of the Drosophila fushi tarazu segmentation gene, Science 1991;252:848-50.

Westwood JT, Clos J, Wu C. Stress-induced oligomerization and chromosomal relocalization of heat-shock factor, Nature 1991;353:822-7.

Sarge KD, Zimarino V, Holm K, Wu C, Morimoto RI. Cloning and characterization of two mouse heat shock factors with distinct inducible and constitutive DNA binding ability, Genes & Dev 1991;5:1902-11.

Becker, PB, Wu C. Cell-free system for assembly of transcriptionally repressed chromatin from Drosophila embryos, Mol Cell Biol 1992;12:2241-9.

Lis J, Wu C. Heat shock factor. In: Yamamoto KR, McKnight SL, eds. Transcriptional regulation. New York: Cold Spring Harbor Press, 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05264-11 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of IAP Proviruses Expressed in Normal and Transformed B-Cells

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

K. K. Lueders	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 MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	-

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☐ (a1) Minors
☐ (a2) Interviews

B

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

It is not known whether transcriptional activation of individual IAP elements is random or if particular elements are activated in different cells because of availability of factors that favor expression of those elements. We have compared sequences of active IAP elements from normal and transformed B-cells to address this question. Mouse plasmacytomas generally express high levels of IAP RNA. We have characterized the IAP genes expressed in a myeloma to determine whether the same IAP genes that are expressed in B-cells are expressed at higher levels, or whether new IAP genes are activated. Previous work has established that a limited and highly characteristic set of IAP elements (designated LS) is expressed in normal mouse B-cells (Kuff AR 91). None of 50 IAP cDNAs isolated from the MPC11 myeloma was of the B-cell type by hybridization to LS IAP specific probes. Twenty-two MPC11 cDNA LTRs were sequenced and found to represent two closely related IAP classes. Their sequences differed markedly from those of the LS IAP class. The IAP cDNAs expressed in this myeloma are very similar to one another in the U3 region of the LTR, where sequences that control transcription are located. These results suggest that expression of IAP elements in transformed cells is not due to random activation, but may be determined by cell specific factors that interact with particular sequence variations within the IAP control regions.

Project Description

Objectives:

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

Major Findings:

Characterization of IAP Elements Expressed in a Myeloma

Characterization of expressed IAP elements has been carried out to determine the basis for selection of particular IAP elements for expression. IAP genes expressed in lymphocytes of BALB/c mouse (lymphocyte specific or LS elements) have been shown to represent a restricted set of elements that can be distinguished by specific sequences in the LTR (Kuff, AR 91). Since myelomas generally express high levels of IAP RNA and can be considered the transformed counterpart of B-lymphocytes, we have determined whether the same (LS) IAP elements are expressed at higher levels after transformation of the cells, and/or whether new IAP genes are activated. Using a MPC11 myeloma cDNA library in lambdaphage gt10 (from Michael Kuehle, NNMC), 50 plaques reacting with a general IAP LTR probe were isolated. Inserts were amplified from phage suspensions by PCR with gt10 primers and tested for the presence of LS sequences by hybridization of LS oligonucleotide probes to DNA on blots. No LS IAP elements were found among this sample.

LTRs of the myeloma IAP cDNAs were sequenced in 22 of the clones, and found to represent two very closely related IAP classes on the basis of their U3 regions, where sequences that control transcription are located. The myeloma cDNAs shared 98-100% homology with their class members. IAP LTRs from a variety of cell types on average share 94% homology. The two myeloma cDNA classes were distinguished primarily in the enhancer 2 region, which has been shown to be a binding sequence for transcription factor EBP-80 (see Kuff, AR 91). In 11 of the clones this region has homology with the SV40 enhancer core motif. In the other 11 cDNAs there is a variant sequence in the enhancer 2 region that has been found in many expressed or transposed IAP elements, and appears to confer higher promoter activity. Both cDNA classes closely resemble an IAP element that had transposed into the IL6 gene in this myeloma, and have other characteristic sequence features that distinguish them from randomly selected IAP elements.

Expression of IAP Elements in other Myelomas

Analysis of poly(A) RNAs from 8 myelomas on Northern blots was used to determine if the pattern of IAP element expression in MPC11 is also a feature of other myelomas. An oligo probe specific for 3 of the 22 MPC11 cDNAs (designated T1) detected a 5.4 kb IAP RNA (derived from the major deleted class of IAP elements) in MCP11. The same size RNA was expressed in 4 other myelomas. Two of the myelomas did not express T1 sequences.

The myeloma Northern blot was hybridized to each of the three LS element probes to determine the expression pattern of these IAP elements. The LS1 probe gave no signal for any of the myelomas. The LS2 probe reacted with the 5.4 kb IAP

RNA (the major RNA in B-cells) in all myelomas. LS3 probe reacted with both 7.2 and 5.4 kb IAP RNAs in all of the myelomas.

These experiments showed that although two of the LS family genes are expressed in MPC11 myeloma, the RNA levels are low, consistent with failure to identify LS cDNA clones in the MPC11 library. LS RNAs are expressed at a higher level in some other myelomas. Results from cDNA sequencing in MPC11 myeloma and from analysis of other myeloma RNAs on Northern blots suggest that expression of IAP elements in transformed cells is not due to random activation of a wide variety of IAP elements, but may be determined by cell specific factors that interact with particular IAP transcription control motifs.

Publication:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05265-10 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

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

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Protein Biochemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.75	2.75	0

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☐ (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. We have found that modification of chromaffin cells with pertussis toxin causes a 40-50% decrease in the amount of cytoskeletal actin. This depolarization of F-actin may account for the increased secretory activity of pertussis toxin-modified chromaffin cells. This decrease in F-actin appears to be independent of changes in known second messengers and may result from a direct interaction of a G-protein with the cytoskeleton. To our knowledge this is the first report that modification with pertussis toxin can reduce the level of F-actin in an unstimulated cell. We have isolated a cytosolic protein which appears to play an important role in regulated exocytosis. Incubation of digitonin-permeabilized bovine chromaffin cells in the absence of Ca^{2+} results in a loss of both cytosolic proteins and Ca^{2+}-dependent secretion. Addition of these leaked proteins prevents this loss of secretory activity. We have purified a protein from an extract of bovine adrenal medulla which can partially prevent this loss of Ca^{2+}-dependent secretion. Antibody against this protein inhibited the ability of leaked chromaffin cell proteins to prevent the loss of Ca^{2+}-dependent secretion. Sequence analysis showed it to be very similar if not identical to bovine brain 14-3-3 protein, a protein not previously thought to be involved in exocytosis. Secretion of norepinephrine by digitonin permeabilized PC12 cells can be stimulated by the addition of GTP S in the absence of Ca^{2+}. We are continuing to try to determine the mechanism by which GTP S induces secretion in these cells and are examining the possible role of nucleoside diphosphate kinase in this response.

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 for the most part have not been 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+}, ATP, GTP, the cytoskeleton, and protein phosphorylation in secretion. While permeabilized chromaffin cells and PC12 cells have similar secretory activities, there are significant differences. For example, GTPγS causes a large increase in Ca^{2+}-independent secretion by digitonin-permeabilized PC12 cells , but it has only a very modest effect on digitonin-permeabilized chromaffin cells.

Major findings:

Modification of a pertussis toxin sensitive G-protein depolymerizes cytoskeletal F-actin: Other investigators have reported that modification of bovine chromaffin cells with pertussis toxin increases both nicotine-stimulated catecholamine secretion from intact cells and Ca^{2+}-dependent secretion from permeabilized cells. Since this enhancement of secretory activity does not appear to result from the release of known second messengers, Aunis, Bader, and coworkers have proposed that pertussis toxin modifies a G-protein which is somehow directly involved in secretory response. We found that

modification of chromaffin cells with a low concentration of N-ethylmaleimide (NEM) has the same effect on secretory activity as modification with pertussis toxin. This effect of NEM appears to result from its reaction with pertussis toxin sensitive G-proteins. When proteins from control cells are incubated with pertussis toxin and [^{32}P] NAD, several proteins are ADP-ribosylated. However, when proteins from cells preincubated with a low concentration of NEM are incubated with pertussis toxin and [^{32}P] NAD, these G-proteins are not ADP-ribosylated, which suggests that they were modified in the cell by NEM. Enhancement of catecholamine secretion by NEM is not additive with that caused by pertussis toxin, and it does appear to result from the release of known second messengers. We found that modification of chromaffin cells with pertussis toxin or with NEM causes a 40-50% decrease in the amount of cytoskeletal F-actin. These decreases in F-actin, like the increases in secretory activity, do not appear to result from the release of known second messengers. Chromaffin cells contain a mesh work of actin filaments just below their plasma membranes. It has been proposed that this mesh work acts as a barrier which inhibits the movement of secretory vesicles to the plasma membrane. Fluorescence microscopy shows that modification with either pertussis toxin or NEM greatly reduces the amount of F-actin in these cortical filaments. If the cortical actin filaments form a barrier which inhibits vesicle movement, then the decreases in cytoskeletal F-actin caused by NEM and pertussis toxin could account for the increased secretory activities of these modified cells. Depolymerization of the cortical actin filaments by either NEM or pertussis toxin would allow a larger fraction of the secretory granules to move close to the plasma membrane in the resting cells. When these modified cells are stimulated by nicotine or K^+-depolarization, a larger number of vesicles would be available for fusion, resulting in an increase in the initial rate of exocytosis. Indeed, the biggest difference in the rates of NE release from control and NEM-modified cells occurred during the first 30 seconds of stimulation. To our knowledge this is the first report that modification with pertussis toxin can reduce the level of F-actin in an unstimulated cell. This decrease in F-actin may result from a direct interaction of a G-protein with the cytoskeleton.

A role for 14-3-3 protein in exocytosis: Incubation of digitonin-permeabilized chromaffin cells in the absence of Ca^{2+} results in a progressive loss of proteins and secretory activity. When the proteins which leak from the digitonin-permeabilized cells are collected, concentrated, and added back to the incubation buffer, this loss of Ca^{2+}-dependent catecholamine secretion is largely prevented, indicating that cytosolic proteins are involved in the secretory response. As only a very small quantity of proteins can be collected from digitonin-permeabilized cells, we used an extract of bovine adrenal medulla as a starting material for the isolation of cytosolic proteins involved in Ca^{2+}-dependent secretion. Fractionation of this extract showed that more than one cytosolic protein is involved in preventing the loss of Ca^{2+}-dependent

secretion. We have purified one of these proteins. Antibody against this protein inhibited the ability of leaked chromaffin cell proteins to prevent the loss of Ca^{2+}-dependent secretion. Sequence analysis showed it to be very similar if not identical to bovine brain 14-3-3 protein, a protein not previously thought to be involved in exocytosis. These results demonstrate that 14-3-3 protein makes a significant contribution to the ability of leaked chromaffin cell proteins to maintain secretory activity. After this work was completed, Morgan and Burgoyne (1992, *Nature* 355, 833-36) reported the identification of two cytoplasmic proteins, Exo 1 and Exo 2, in extracts of sheep brains which can partially reactivate secretion in permeabilized bovine chromaffin cells. Amino acid sequences of two peptides from Exo 1 suggest that it may be a form of sheep brain 14-3-3 protein. These authors did not report the effects of anti-Exo-1 antibody. While our results and those of Morgan and Burgoyne suggest that 14-3-3 protein is involved in Ca^{2+}-dependent secretion in permeabilized chromaffin cells, its precise role is not known. While 14-3-3 protein has been shown to activate both tyrosine and tryptophan hydroxylases, it is unclear how stimulation of these hydroxylases could account for an increase in Ca^{2+}-dependent catecholamine release. Also, the presence of 14-3-3 protein in tissues which do not contain detectable amounts of either tyrosine or tryptophan hydroxylases suggests that it has some more general function. An inhibitor of protein kinase C isolated from sheep brain also appears to be a form of 14-3-3 protein. It is unlikely that the effect of adrenal 14-3-3 protein on secretion in permeabilized chromaffin cells results from its inhibiting protein kinase C, as other inhibitors of protein kinase C either decrease or have no effect on Ca^{2+}-dependent secretion in these cells, and activation of protein kinase C enhances Ca^{2+}-dependent secretion. We have started to try to identify what other proteins or structures might interact with 14-3-3 protein. Two obvious possibilities, secretory vesicles and F-actin, do not appear to bind adrenal 14-3-3 protein.

Stimulation of secretion by ATPγS and GTPγS: Secretion of norepinephrine by digitonin-permeabilized PC12 cells can also be stimulated by the addition of GTPγS or GMP-PNP 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. We are continuing to try to determine the mechanism by which GTPγS induces secretion in these cells. We have previously reported that ATPγS can also stimulate Ca^{2+}-independent secretion of norepinephrine by digitonin-

permeabilized PC12 cells. While our initial results suggested that this stimulation by ATPγS does not result from its conversion into GTPγS, our more recent data suggest that the effect of ATPγS may in part result from its conversion into GTPγS by nucleoside diphosphate kinase. If this conversion does occur, the resulting GTPγS must be produced in close proximity to the GTP-binding protein responsible for stimulating secretion, as GTP is a poorer inhibitor of secretion evoked by the addition of ATPγS than that evoked by the addition of GTPγS.

Publications:

Wu YN, Wagner PD. Effects of phosphatase inhibitors and a protein phosphatase on norepinephrine secretion by permeabilized bovine chromaffin cells, Biochim Biophys Acta 1991;1092:384-90.

Yang YC, Vu ND, Wagner PD. Guanine nucleotide stimulation of norepinephrine secretion from permeabilized PC12 cells: effects of Mg^{2+}, other nucleotide triphosphates and N-ethylmaleimide, Biochim Biophys Acta 1992;1134:285-91.

Wu YN, Yang YC, Wagner PD. Modification of chromaffin cells with pertussis toxin or N-ethylmaleimide lowers cytoskeletal F-actin and enhances Ca^{2+}-dependent secretion, J Biol Chem 1992;267:8396-403.

Wu YN, Vu ND, Wagner PD. Anti-14-3-3 protein antibody inhibits stimulation of secretion by chromaffin cell cytosolic proteins, Biochem J 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05267-08 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

M. Yarmolinsky	Chief, Microbial Genetics and Biochem. Section,	LB	NCI
D. Chattoraj	Microbiologist	LB	NCI
G. Mukhopadhyay	Visiting Fellow	LB	NCI
S. Sozhamannan	Visiting Fellow	LB	NCI
P. Papp	Visiting Fellow	LB	NCI
M. Lobocka	Visiting Fellow	LB	NCI
H. Lehnherr	Visiting Fellow	LB	NCI (expected July, 1992)

COOPERATING UNITS (if any)
Dr. T. Schneider, NCI, FCRCD, Laboratory of Mathematical Biology;
Dr. Marc S. Lewis, BEIP, NCRR, NIH

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Microbial Genetics and Biochemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.2	6.2	-

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☐ (a1) Minors
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B

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

The mechanisms by which every daughter cell in a growing bacterial culture is assured of an equivalent genetic dowry continue to be the focus of our inquiries. To this end we have been studying an inessential element of the bacterial trousseau, a stable, low-copy number plasmid, prophage P1. During the period of this report studies of plasmid replication have provided considerable molecular detail about how the P1-encoded initiator protein, RepA, and the host-encoded initiator protein, DnaA, perform their functions and where and how the host heat-shock proteins DnaJ, DnaK, and GrpE enter into the picture. Evidence has been obtained that, although they are capable of monomerizing dimeric RepA protein, the function of DnaJ, DnaK and GrpE in vivo is to perform a more subtle alteration. These proteins activate the already monomeric form of RepA for binding to specific sites. Activation for binding appears to be the unique role of the heat shock proteins in P1 replication. Binding of the activated RepA monomers to sites in the P1 origin along one face of the DNA helix causes a right-handed wrapping of the DNA about the protein core and, through a cooperative interaction, enhances the specific binding of initiators to the origin. It is the bound DnaA protein rather than the bound RepA protein that appears to accomplish a strand opening that is presumed to be essential for primer synthesis. Studies of plasmid partitioning during the period of this report have challenged established ideas about the structure and function of the P1 partition module. The sequence of P1 parB (one of two P1 proteins required for active partition) has been corrected, a cryptic incompatibility element has been unmasked, and the interference with stabilization that occurs when two plasmids possess the same partition module (partition incompatibility) has been shown to lead to a greater loss of stability than the commonly held model of such incompatibility admits. In addition, new tools have been developed with which to discern the presently elusive features of DNA context that we find to be critical in determining whether the centromere-analog, parS, is functional or dysfunctional.

Project Description

Objectives:

The aim is to provide an understanding, in molecular detail, of mechanisms that enable an autonomous genetic element to be stably inherited.

Replication

A. Characterization of RepA-Iteron Interactions (in collaboration with T. Schneider)

The P1 plasmid replicon includes 14 highly conserved 18-bp sites (iterons) to which the P1-encoded initiator protein, RepA, can bind. The roles of individual bases within the iterons and the basis for the high degree of conservation were studied by analysis of how mutations in the iterons affect their function in vivo and in vitro. Point mutations in four of eight positions of complete conservation resulted in full loss of binding capacity; in the remaining four the loss due to mutation was partial as it was in several other positions. Footprints of RepA suggest that at the four most critical positions RepA is contacted through the major groove. The other four fully conserved bases are probably involved in central distortion of the B-DNA structure. Additionally evidence was obtained that the affinity of RepA for its binding sites is not the only parameter of the RepA-iteron interaction relevant to function. It appears that the unusual degree of conservation may be dictated by the several roles that each of the binding sites appears designed to play in the activation and control of replication.

B. Mechanism of Stimulation by Heat Shock Proteins DnaJ and DnaK of the Site-Specific DNA Binding by RepA (in collaboration with Marc S. Lewis)

Purified RepA binds to DNA with low affinity and, at elevated protein concentrations, forms extensive DNA aggregates due to nonspecific binding. Escherichia coli heat shock proteins DnaJ and DnaK, previously implicated in P1 plasmid replication, were found to prevent aggregate formation in an ATP-dependent fashion and to stimulate specific binding. Analytical ultracentrifugation revealed that RepA was in monomer-dimer equilibrium with a K_D of 3 μM in our binding buffer. We suggest that the dimeric form is responsible for the aggregates seen at elevated RepA concentrations and disaggregation under the influence of DnaJ and DnaK involves breakdown of dimers into monomers. Since stimulation of specific binding is also seen at physiological protein concentrations that are far below the K_D, the in vivo action of the heat shock proteins in promoting replication most likely involves refolding of inactive monomers to an active form rather than RepA monomerization.

C. Role of the Heat Shock Proteins in vivo

It was shown previously that the defect in the autorepression function of RepA in ΔdnaJ cells can be overcome by overproduction of wild type RepA or, without overproduction, by certain mutant RepAs. Of RepA mutants selected solely for increased affinity of RepA to its binding sites, 11 of 12 also regained replication proficiency. Such studies have been extended to ΔdnaJ-K, and ΔgrpE

mutant strains with similar results. It can be concluded that the only role of the heat-shock proteins DnaJ, DnaK and GrpE in P1 plasmid replication is to improve specific DNA binding of RepA. The mutations that conferred heat-shock independence mapped in the central region of the repA orf spanning only 46 codons. The corresponding region of the protein is therefore involved in DNA binding either directly or indirectly by helping to fold RepA appropriately for DNA binding.

D. Requirement for Strand-Opening of P1 Plasmid Origin DNA

The P1 plasmid origin binds the plasmid-encoded initiator, RepA, at five sites and the host-encoded initiator, DnaA, at two. Both proteins and binding sites are required for replication of the plasmid. Their role in strand-opening of the origin was investigated since this reaction is considered essential in the synthesis of the first primer. Binding of RepA alone to the origin led to wrapping of the DNA around the protein in a right-handed sense. Although a compensatory turn that unwinds a region of the DNA was expected, there was no evidence of strand-opening in the origin when probed with a reagent, $KMnO_4$, specific for single-stranded DNA. In contrast, limited strand-opening was evidenced in the presence of DnaA alone. Addition of RepA stimulated the reaction. In separate experiments, addition of DnaA enhanced specific DNA binding activity of RepA. It appears that a role of DnaA in P1 plasmid replication is in strand-opening of the origin, a reaction that may require a cooperative interaction with RepA.

Partition

A. Revisions in the Concept of Partition Incompatibility

Incompatibility, a central concept in plasmid biology, is the failure of plasmids with related maintenance functions to be stably coinherited in the absence of selection pressure. Incompatibility has been attributed to a competition among plasmids for participation in a process that results in their stable maintenance and for which they are selected at random. Does this simple competition model explain the incompatibility that is associated with a shared mechanism of plasmid partition? We suggest not, in part on the basis of the following evidence.

Experiments were performed in which the stability of two replicons that are themselves compatible, but each bearing intact P1 partition modules (parA$^+$B$^+$S$^+$), were examined following release from selection pressure. The results of these experiments showed that the separately stable plasmids engaged, when together, in a more severe plasmid destabilization than could be accounted for by a null partition defect. Partition incompatibility appears to have an aggressive component that has not been previously recognized.

Surprisingly, deletion of parSmin (the region of parS that is considered to be essential for partition function) from one of the two plasmids did not eliminate its susceptibility to partition incompatibility. The locus or loci responsible for this incompatibility is being determined by mutational analysis of the residual partition region. This analysis is being used to generate a plasmid capable of furnishing functional par proteins without interference by the previously undetected incompatibility element.

B. Improved System for Studying par Gene Function

In a pcnB mutant of E. coli the copy number of pBR322 is specifically reduced to a value that allows both stabilization and destabilization of the plasmid to be assessed within a few bacterial generations. The host mutation does not interfere with expression or function of the P1-encoded par genes. Thus the familiar vector pBR322 can be used as a convenient reporter of partition function.

In E. coli pcnB, a modest destabilization of pBR322-parS by the presence of a source of ParB protein was shown to depend on the particular DNA context of parS and not on a high unphysiological concentration of ParB, as was previously assumed. Effects of DNA context on the function of parS forms an important part of the systematic study that is described in the next section.

C. Selection of Mutants Affected in par Function

The search for mutants altered in par function has been impeded by the inherent weakness of the par phenotype (decreased plasmid stability) and the diversity of irrelevant mutations that can mimic the par phenotype. Our finding that modest expression of the P1-encoded par proteins (ParA and ParB) or ParB alone can cause a drastic instability of a pSC101-based vector when it carries the P1 centromere-analog, parS, has permitted a direct selection for mutations in various components of the partition module. Of those mutations that have been studied so far, certain ones have been mapped to parSmin; others have been mapped to adjacent regions of the vector. The location of some of the mutations in the neighborhood of parS attest to the profound influence of context on parS function.

D. Revision of the P1 parB Sequence; Comparative Studies

Two errors in the published parB gene sequence were detected. Correction of these errors truncates the parB open reading frame by 10 codons, thereby conveniently relocating a presumed ParB binding site to a position outside the parB gene itself and disposing of an apparent region of non-homology between the parB gene of P1 and that of several closely related plasmids.

A computer search for sequences related to P1 par sequences revealed the presence of a parS-like sequence in a virulence plasmid from Salmonella. Clones of the upstream region were obtained and the presumptive par genes are being sequenced. This and other recent evidence suggests that the genes adopted by P1 for active plasmid partition are widely distributed in nature.

Publications:

Funnell B. The P1 plasmid partition complex at parS: the influence of Escherichia coli integration host factor and of substrate topology, J Biol Chem 1991;266:14328-37.

Yarmolinsky MB, Lobocka MB. Bacteriophage P1. In: O'Brien SJ, ed. Locus maps of complex genomes. 6th ed. New York: Cold Spring Harbor Laboratory Press, 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05268-05 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

M. Lichten	Senior Staff Fellow	LB	NCI
T-C. Wu	Chemist	LB	NCI
J. McCarthy	Staff Fellow	LB	NCI
M. Daly	IRTA Fellow	LB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Developmental Biochemistry and Genetics

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	3.4	-

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

B

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

We continued research on the molecular mechanism of meiotic recombination in the yeast Saccharomyces cerevisiae. We have focused on an initiating event in meiotic recombination, the formation of double-strand DNA breaks, with an aim towards determining the factors that control the level and position of these breaks, and isolating the enzyme(s) responsible for their formation. In addition we have analyzed several short regions of the yeast genome that contain sequences that stimulate both meiotic recombination and double-strand DNA breaks.

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. Position Effects in Meiotic Recombination

We (Michael Lichten and Carol Wu) have shown that the level of meiotic recombination in a genetic interval is affected by its genomic position. Diploid yeast strains that contain either of two tester genes (LEU2 or ARG4) inserted at one of several loci display a wide range in frequencies of meiotic recombination within those genes, even though the intervals examined were flanked at each insert location by substantial regions of sequence identity. These results indicate that elements are present in the yeast genome that modulate levels of meiotic recombination by acting over considerable distances in a target gene-independent manner. By examining the effect of various plasmid inserts at the HIS4 locus on recombination in adjacent genes, we have shown that these effects can be transmitted over at least 20 kb of flanking chromosomal sequences. We are currently further examining the nature of these position effects and determining the distances over which they are active.

B. Factors That Control the Level of Meiosis-Induced Double-Strand DNA Breaks

We have previously shown that the formation of double-strand DNA breaks (hereafter referred to as DSB) during meiosis does not simply involve the recognition and cutting of a short nucleotide sequence. A DSB occurs in the ARG4 promoter region at the normal ARG4 locus, but not in the same region inserted at various other loci in the context of a pBR322-based plasmid. Double-strand breaks do occur at other places in these plasmid inserts, primarily at the ARG4-pBR322 junction. Insert loci that display high frequencies of ARG4 recombinants also suffer the highest level of DSB; insert loci with lower frequencies of recombination display correspondingly lower amounts of DSB. Thus, levels of meiotic recombination within the ARG4 gene and levels of DSB just outside this gene are determined, at least in part, by the same factors. Carol Wu has also shown that the insertion of sequences with high levels of DSB suppresses DSB at sites in neighboring regions. Remarkably, this suppression is seen not only in cis (on the chromosome harboring the insert) but also in trans (on homologous chromosomes without the insert). This observation raises the intriguing possibility that homologous chromosomes become paired in meiosis prior to the time that DSB are formed. We plan to look for direct physical evidence of such an early association, using intra- and interstrand DNA crosslinking reagents combined with 2-dimensional electrophoresis.

C. Chromatin Structure Determines the Location of Meiosis-Induced Double-Strand DNA Breaks

The results described above allow the suggestion that aspects of chromosome structure other than primary DNA sequence play an important role in determining the location of DSB. Carol Wu has tested this suggestion by examining the relationship between meiosis-induced double-strand breaks and chromatin structure. To date, we have examined two regions of the yeast genome. In both regions, we observe a one to one correspondence between meiotic DSB sites and sites in chromatin that display elevated sensitivity to DNase I. This finding is consistent with the suggestion that the locations of meiotic DSB are primarily determined by the accessibility of DNA in chromatin to a non-specific endonuclease. Preliminary experiments (Carol Wu and James McCarthy) indicate that such a nuclease is present in preparations of yeast nuclei. We plan to purify and characterize this nuclease activity, and also examine the relationship between DSB and chromatin structure in other regions of the yeast genome.

D. Sequences That Modulate the Level of Meiotic Recombination

Michael Daly has further characterized two fragments of yeast DNA that appear to contain portable stimulators of meiotic recombination. The elements contained in these fragments act in *cis* to promote both gene conversion and double-strand breaks in flanking sequences to both sides of the fragments. We have determined the DNA sequence of both of these ca. 3 kb fragments; no obvious regions of similarity were observed. This sequence information will, however, be useful in subcloning experiments designed to further localize these "enhancer" elements.

Publication:

Wu TC, Lichten M. Position effects in meiotic recombination. In: Cooper GM, Haseltine FP, Heyner S, Straus JF, eds. Meiosis II: contemporary approaches to the study of meiosis. AAAS, Washington, D.C., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05271-01 LB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Regulation and Function: The bZIP Proteins

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

C. Vinson	Senior Staff Fellow	LB	NCI
S. Boyd	Biologist	LB	NCI

COOPERATING UNITS *(if any)*

Kelly Thompson and Ernesto Friere, Julie Johnson and Tom Tullius, Johns Hopkins University; Mark Lewis, NIH

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Microbial Genetics and Biochemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

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

☐ (a1) Minors B

☐ (a2) Interviews

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

The last several years have seen the identification of a large number of sequence-specific DNA binding proteins that are important for gene regulation. These proteins use a small number of DNA binding motifs to interact with DNA. This laboratory is focusing on two related motifs that interact with DNA in a sequence-specific manner, the bZIP and the bHLH-Zip motifs. Both motifs bind as dimers to abutted dyad symmetric DNA sequences. We have proposed molecular models for both motifs. We are testing aspects of our models using site directed mutagenesis and chemical probing of DNA structure.

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

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

Project Description

Objectives:

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

Major Findings:

A. The bZIP Motif

1. Novel dimerization partners of bZIP members have been determined

Using simple interhelical salt bridge rules, we have predicted novel dimerization partners for several bZIP molecules. We suggested that the bZIP molecules ATF4 and IGEBP1 would form heterodimers. We have requested and received plasmids that encode these proteins from the researchers who initially cloned these molecules and have generated pure samples of both proteins. As predicted, they prefer to form heterodimers. We are presently quantitating our results. Additional experiments suggest that ATF4 can form heterodimers with three of the C/EBP family of bZIP molecules.

2. A "Dominant Negative" form of C/EBP can be generated

Again, using simple rules of interhelical salt bridge formation, we have designed derivative molecules of C/EBP that dimerize with wild type C/EBP better than C/EBP interacts with itself. This result suggests that we are developing a rich understanding of the rules that govern dimerization specificity. Biologically, the intriguing possibility is that we will be able to introduce these "Dominant Negative" molecules into cells and disrupt normal C/EBP function. These studies will presently be continued.

3. Leucine zipper dimerization is enthalpically driven

We are using biophysical methods to determine the nature of the forces that are important for dimer formation. These experiments are being conducted using an analytical ultracentrifuge in collaboration with Mark Lewis at the NIH. Our initial studies, using a 63 amino acid recombinant protein generated by Jon Shuman at the University of Alabama, show that this molecule is in a monomer-dimer equilibrium with a dimer dissociation of 10 nm. An analysis of dimerization vs. temperature suggests that the driving force for dimer formation is enthalpic. We plan on continuing these studies with our designed molecules that have different dimerization specificities. Initial calorimetric studies with Kelly Thompson in Ernesto Friere's group at Johns Hopkins University confirm and compliment the analytical ultracentrifuge data.

B. The bHLH-Zip Motif

The last several years have seen an explosion in the identification of sequence-specific DNA binding proteins. These proteins use a small number of structural motifs to interact with DNA. One of these motifs is called the bHLH-Zip motif and includes the oncogenes myc/mas and the determination proteins myoD/E12. The

large number of proteins that have this motif reveals the conserved seminal structural elements that are critical for function. Using this comparative molecular biological approach, we have generated a structural model for the interaction of the bHLH-Zip protein interacting with DNA. This work is presently in press. The structural model makes several strong predictions concerning which amino acids are interacting with which conserved DNA elements that all bHLH-Zip proteins bind. We have generated mutations in the predicted amino acids that are critical for DNA binding and are presently using a variety of chemical modifications of DNA methods to examine the role of the amino acids. This work in being conducted in collaboration with Julie Johnson who is in Tom Tullius' laboratory at the Johns Hopkins University.

Publication:

Vinson C, Garcia KC. Molecular model for DNA recognition by the family of basic-helix-loop-helix-zipper (bHLH-Zip) proteins, The New Biol, 1992, in press.
Vinson C. Amphipathic helices in proteins that bind to DNA: The bZIP and bHLH-Zip DNA binding motifs. In: Epand RM, ed. The amphipathic helix. CRC Press, 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05272-01 LB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Structure and Function of RNA Polymerase II

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

M. Mortin Senior Staff Fellow LB NCI

COOPERATING UNITS *(if any)*

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

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	-

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A large number of genes required for Drosophila development function by regulating the transcription of other genes. I am interested in the connection between the basic transcription machinery and pattern formation. My work shows that specific mutations in RNA polymerase II (polII) itself can disrupt different discrete steps in developmental processes. Many of the developmental defects caused by polII alleles mimic those elicited by loci assumed to encode transcription factors. This observation suggests that polII plays an active role in choosing which genes to transcribe, possibly by interacting either directly or indirectly with developmentally regulated DNA binding proteins.

To fully understand development it is essential to define the interactions between regulatory proteins and polII. In order to accomplish this goal I have begun to identify mutations that interact with existing mutant alleles of polII. I have isolated mutations in other genes (extragenic) that either enhance or suppress mutant phenotypes elicited by certain polII alleles. These might encode proteins that interact functionally with polII and will include: 1) previously undescribed subunits, 2) proteins required for modification of polymerase, 3) proteins that regulate gene expression during development, and 4) proteins that maintain chromatin structure or nuclear architecture. The primary goal of this research is to elucidate the mechanism by which transcriptional regulation controls development. This will be accomplished by the determination of the protein products encoded by genes identified as enhancer and suppressor mutations and the defining of the molecular basis of the interactions between these and known subunit mutations.

Project Description

Objectives:

1. To identify the sequence changes in seven alleles of the second-largest subunit of RNA polymerase II responsible for suppression of a conditional lethal elongation defective mutation in the largest subunit.

2. To clone two genes that were identified as suppressors of the elongation defective mutation mentioned above but that are thought not to be subunits of RNA polymerase II and begin to define their relationship to one another.

Major Findings:

During the past year I have completed and had accepted for publication a manuscript describing a direct selection of mutations in the second-largest subunit of RNA polymerase II. A second manuscript describing the discovery of a mutation in the ninth-largest, which happens to map in the same region as the second-largest subunit, was recently published. A collaboration with Y. Chen and A. Greenleaf at Duke University has been established to begin mapping mutations within the two largest subunits. A combination of PCR, conformational polymorphism detecting gels and sequencing will be used to map putative point mutations.

I have isolated 41 second-site suppressors of mutations in the two largest subunits of polII. Twelve of the 20 that have been mapped are extragenic, identifying at least four genes. The 21 remaining suppressor mutations are likely to identify more new genes. Seven suppressor mutations of a conditional lethal allele of the largest subunit map to the second largest subunit of polII. These will be sequenced as described above. Other genes identified as second-site suppressor mutations are also likely to encode proteins involved in transcription.

Two genes, S3 and S8, which suppress an elongation or termination defective mutation in the largest subunit are not thought to be polII subunits and have been targeted for cloning. S8 is epistatic to S3 suggesting a pathway such that S3 acts on the largest subunit of polII first and then this altered structure or complex interacts with S8. I hypothesize that since S8 acts downstream from an elogation defective polII it may behave in a manner analogous to rho factor in bacterial termination. The S3 locus acts upstream suggesting that it modifies the largest subunit of polII. The phosphorylation state of the C-terminal domain of the largest subunit regulates polymerase activity. The S3 protein might act as a kinase or phosphatase. This would suggest that the phosphorylated state of polymerase regulates elongation. I am also collaborating with D. Price (University of Iowa) to determine the effects of this conditional lethal allele in the presence and absence of suppressors using an *in vitro* transcription assay.

I am using an altered transposable element that has both fly and bacterial selectable markers. I can mobilize the transposable element and select for mutations in either enhancer or suppressor loci. These elements can then be cloned along with adjacent genomic fly DNA by plasmid rescue. This is

accomplished by isolating genomic DNA from mutant flies, restricting the DNA with enzymes that cut only once in the vector sequence, self ligating the resulting DNA fragments and transforming directly into E. coli. Starting with this technique and then screening a genomic library, I have cloned and restriction mapped approximately 30kb of genomic DNA surrounding the insertion site of one transposon in or near the S3 locus. I am using the clones I have isolated to delimit the extent of the S3 locus by examining mutations in this gene for alterations in their DNA and will then determine its developmental pattern of expression and isolate cDNA clones for sequencing.

Publications:

Harrison DA, Mortin MA, Corces VG. The RNA polymerase II 15 kilodalton subunit is essential for viability in Drosophila, Mol Cell Biol 1992;12:928-35.

Mortin, MA, Zuerner R, Berger S, Hamilton BJ. Mutations in the second-largest subunit of Drosophila RNA polymerase II interact with ubx, Genetics 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 05273-01 LB

PERIOD COVERED: October 1, 1991 to September 30, 1992

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

Molecular Genetics of Sexual Dimorphism

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

D. H. Hamer	Chief, Gene Structure and Regulation Section	LB	NCI
N. Hu	Visiting Fellow	LB	NCI
J. Zeng	Visiting Fellow	LB	NCI
A. Pattatucci	NRC Fellow	LB	NCI

COOPERATING UNITS *(if any)*

Jeremy Nathans, Johns Hopkins Medical School; Larry Charnas, Human Genetics Branch, NICHD

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Gene Structure and Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	2.00	.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☒ (a2) Interviews

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

A project to study the molecular basis for the sexual differentiation of neural structure and function has been initiated. Strategies are being developed to isolate and characterize genes that contribute to sexually dimorphic behavior in organisms as diverse as Drosophila, rats and man.

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

Projection Description

Objectives:

To understand the molecular genetics of sexually dimorphic brain differentiation.

Major Findings:

A. Human Studies

Human sexual behavior is variable. Although the majority of individuals are oriented towards members of the opposite gender, a minority prefer members of the same gender. Twin and adoption studies suggest that a substantial fraction of this variability is hereditary. We propose to critically examine this hypothesis by using the tools of modern human genetics.

1. PEDIGREE SEGREGATION ANALYSIS. Family histories are being collected from a series of homosexual men and women. Information is also obtained on Kinsey scale measures, age of acknowledgment for sexual orientation, and reproductive rates. This data will be analyzed by standard statistical methods to determine the heritability, number, and mode of transmission for putative sexual orientation genes.

2. CANDIDATE GENE ANALYSIS. The testis determining factor encoded by the Y chromosome is the primary sex determinant in mammals. Subsequent sexual development is driven by the presence or absence of testosterone synthesized by the testis. Therefore the gene for the testis determining factor, SRY, and loci that encode gonadal steroid metabolizing and transducing proteins are logical candidates for involvement in sexual orientation. We have established PCR and denaturing gradient gel methods to probe for point mutations in both the SRY gene and, in collaboration with Dr. Jeremy Nathans at Johns Hopkins University, the androgen receptor gene. DNA samples from 130 unrelated male and female homosexuals have been prepared for analysis by these methods.

3. LINKAGE ANALYSIS. Genes that contribute to the development of any human trait can, in principle, be detected by linkage studies, even when the identity and function of these genes are unknown. The critical reagents for such studies are families in which there are multiple individuals who express the trait. We are in the process of recruiting, interviewing and obtaining DNA from families in which there are multiple individuals with homosexual orientation. To date, 38 families with an appropriate structure for linkage analysis have been identified.

4. HIV SUSCEPTIBILITY AND ALCOHOLISM. Sexually active homosexual men are at increased risk for infection with HIV, the virus that causes AIDS, and both gay men and lesbians have been reported to have increased rates of alcoholism and substance abuse. There is circumstantial evidence that susceptibility to both certain outcomes of HIV infection, such as Kaposi's sarcoma, and to alcohol abuse are genetically affected, but the relevant genes have not been identified. We propose to detect such genes by linkage and association studies. In collaboration with physicians at the NIAID outpatient HIV Clinic and the NIAAA

Inpatient Alcoholism Clinic, we have recruited several subjects with family histories of both homosexual orientation and HIV infection or alcoholism, and will analyze DNA from these individuals and relevant family members for genes encoding histocompatibility antigens, viral receptors and the dopamine receptor.

B. Courtship Behavior in Drosophila

The fruitfly Drosophila melanogster displays an elaborate and stereotypical courtship ritual that involves chemosensory, auditory and visual components. We have initiated a P-element screen of the 2nd and 3rd chromosomes for mutations that alter this behavior in a sex-specific manner. We will be particularly interested in insertions in the region of fruitless, a complex chromosomal inversion that leads to male-male courtship.

C. Sexually Dimorphic mRNAs in the Rat Hypothalamus

In rats, adult sexual behavior is profoundly influenced by the activity of gonadal steroids during a short critical period of prenatal and postnatal development. Neuroanatomical observations show that the sexually dimorphic nucleus of the preoptic area of the hypothalamus (SDN-POA) is an important target for neurosteroid action during this eight day period; interestingly, a corresponding region in the human brain has been associated with male homosexuality. We will attempt to clone sexually-dimorphic and/or androgen-inducible mRNAs from this region using PCR-mediated subtractive hybridization of hypothalmic RNA from male versus female rats and/or adrogen-treated versus control castrated animals.

Publication:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05274-01 LB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

IAP Proviruses as Multilocus Probes for Mapping on Mouse Chromosomes

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

K. K. Lueders	Research Chemist	LB NCI
E. L. 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 MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	-

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 mouse is recognized as an important tool for generating high resolution genetic linkage maps. Multilocus probes derived from endogenous proviral sequences have been particularly informative in establishing reference loci. Such maps are useful for characterizing and mapping human disease genes because of conserved synteny and gene function between mouse and human. Oligonucleotide probes based on distinctive sequences in the LTRs of IAP elements expressed in B-cells (LS elements) react with 15-30 restriction fragments representing proviral/cell DNA junctions in mouse DNA. These oligos detect characteristic polymorphic strain distribution patterns (SDPs) in the DNAs of different inbred strains of mice, and thus provide useful multilocus probes for gene mapping. Chromosomal assignments have been established for 41 LS IAP loci by comparing their SDPs with those of known genetic markers in recombinant inbred strains of mice.

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

Project Description

Objectives:

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

Major Findings:

Chromosomal Mapping of IAP LS Elements

Previous work has established that a limited and highly characteristic set of IAP elements (designated LS) is expressed in normal mouse B-cells, and that LS IAP oligo probes detect a limited subgroup of the total IAP elements in the mouse genome. These probes detect restriction fragments with characteristic strain distribution patterns (SDPs) that are polymorphic among inbred strains of mice (see Kuff AR91). For chromosomal mapping, individual IAP proviruses were analyzed as HindIII restriction fragments in recombinant inbred (RI) strains of mice. RI mice contain a random mix of progenitor genomes, and since linked genes have similar SDPs, DNA from these mice can be used for gene mapping. Extensive linkage maps have been established for RI strains of mice using a variety of polymorphic markers that differ in their SDPs. We have established chromosome assignments for 41 IAP loci by comparing their SDPs with those of known genetic markers in RI strains of mice. One provirus that is hypomethylated in the thymus of three strains of mice and in three myelomas is closely linked to the met oncogene on chromosome 6. Another commonly hypomethylated provirus is linked to a testis specific cDNA mapping to chromosome 17. These proviruses could be markers for regions of mouse chromosomes that are constitutively transcriptionally active.

Other element specific oligo probes have been derived from subfamilies of IAP elements expressed in plasmacytomas. Chromosomal mapping using backcrosses between M. musculus and M. spretus mice is also in progress, and should permit us to map additional IAP elements that are not polymorphic among laboratory strains of mice.

Publication:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08212-18 LB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

From Gene to Protein: Structure, Function, and Control in Eukaryotic Cells

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

S. L. Berger	Chief, Genes and Gene Products Section	LB	NCI
R. E. Manrow	Senior Staff Fellow	LB	NCI
D.W. Batey	IRTA Fellow	LB	NCI
A. De la Rosa	Visiting Fellow	LB	NCI
G. Kurys	IRTA Fellow	LB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Genes and Gene Products Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.50	4.50	-

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

Note: 25% of this effort is AIDS-related

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

Prothymosin α genes, pseudogenes, mRNA transcripts and proteins have been investigated with the following results: The gene family consists of six members, five of which can be classified as processed pseudogenes based on complete sequencing of the genes and their flanking regions. Although the pseudogenes possess consensus regulatory signals for transcription and translation and, in some cases, highly conserved open reading frames, none is expressed. The intron-containing gene gives rise to two transcripts by an unusual form of alternative splicing. As a result of nonconsensus splice acceptor selection at two adjacent GAG triplets, the second triplet, which would be expected to be exonic, is sequestered within the adjacent intron to generate the common form of prothymosin α mRNA. The rare form (~10% of prothymosin α mature mRNA in all tissues examined) contains the triplet and encodes an identical protein made one residue longer by the insertion of a glutamic acid residue at position 39. These experiments have led to a refinement of the rules for splice acceptor selection. The mature protein is posttranslationally modified. The adduct has been identified and modified peptides have been isolated. Although experiments with synchronized human myeloma cells treated with antisense oligomers have shown that prothymosin α is required for cell division, modification of the protein(s) occurs throughout the cell cycle. Advances in understanding the function of prothymosin α in cell proliferation have been made as a consequence of: (1) devising a model system for use as an assay; (2) engineering a transgenic mouse; (3) fusing prothymosin α with a seven-amino acid epitope; and (4) constructing a CAT vector to investigate the interaction of the gene with c-Myc.

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 α, an abundant, acidic protein found only in the nuclei of proliferating cells of all types. We plan to elucidate the function of prothymosin α.

Major Findings:

Prothymosin α was first thought to be a precursor for thymic hormones and later a thymic hormone itself. However, observations from this laboratory were not consistent with this view: (1) the primary translation product of two different cloned prothymosin α cDNAs lacked an amino-terminal signal peptide; (2) the protein contained a functional nuclear localization signal capable of directing fusion proteins to the nucleus; and (3) prothymosin α synthesized by COS cells in response to transfection with the human prothymosin α gene was situated exclusively in the nucleus. We propose, instead, a role in cell proliferation as a result of our recent experimental findings as follows: (1) prothymosin α gene activity is evident in all tissues examined; (2) the amount of prothymosin α and its mRNA is roughly proportional to the proliferative activity of the tissue from which either is isolated; (3) prothymosin α is induced in normal human lymphocytes upon growth stimulation with mitogens, in serum-starved NIH 3T3 cells subsequent to restitution of serum, or in human myeloma cells during recovery from stationary phase; and (4) division of synchronized, human myeloma cells is reversibly inhibited by treatment with any of four antisense oligodeoxyribonucleotides targeted to different regions of the prothymosin α mRNA. It is also interesting that, to date, activation of the c-Myc protein results in transcriptional activation of only a single gene—prothymosin α.

During the past year we have embarked on three projects aimed at assigning a function to prothymosin α. (1) Because antibodies against prothymosin α have poor

specificity and low titer, we have epitope-tagged prothymosin α with seven amino acids affixed to the amino terminus. The fusion protein can be immunoprecipitated quantitatively with an antibody directed against the epitope. Although construction of the gene and its expression in COS cells were straightforward, the antibody precipitations were not quantitative until we discovered that the epitope, and perhaps prothymosin α as well, must be partially folded for formation of antigen-antibody complexes to occur. (2) In collaboration with Drs. James and Nancy Lee, we have engineered a transgenic mouse in which the prothymosin α gene is expressed behind the CD3 promoter. A strong T-cell promoter was chosen because the thymus is one of the richest sources of prothymosin α in animal tissues. (3) We have also cloned and sequenced approximately 1 kb of the region immediately upstream of the beginning of transcription of the prothymosin α gene and constructed a CAT vector containing 5 kb of upstream sequence. The region contains several myc consensus binding sites. A study of the effect of c-Myc on transcription of prothymosin α with Dr. Chi Dang (Johns Hopkins) as a collaborator is in progress. The results of these efforts, which are marginally beyond the tooling up stage, are too preliminary to report.

Prothymosin α is posttranslationally modified. Previously, another laboratory reported that prothymosin α was covalently attached to a 20 nt RNA. We have been unable to confirm this observation. Our data indicate that prothymosin α is phosphorylated and that the phosphate is unaffected by DNAse or several RNases, but is quantitatively removed by either calf intestine alkaline phosphatase or bacterial alkaline phosphatase. The phosphorylated protein comigrates in two dimensional gels with synthetic prothymosin α prepared by translating synthetic, capped prothymosin α mRNA in wheat germ. Thus, evidence for either an adduct of the size proposed for bound RNA or for a substantive change in charge is lacking. Using thin layer electrophoretic analysis of prothymosin α, which was labeled in cell culture with [^{32}P]orthophosphate and briefly hydrolyzed in acid, we have found that the label associates exclusively with serine residues. Prothymosin α is a highly acidic protein of 13 Kd, the predominant form of which contains 53 carboxyl groups, 7 hydrophobic residues, 2 clusters of basic amino acids each with 5 residues, and no Cys, His, or aromatic residues. There are four serines located at positions 83, 9, 8 and 1; in the mature protein the last is apparently acetylated at the amino terminus after the removal of the initiator formylmethionine residue. In order to devise and test a strategy for isolating peptides, it was necessary to obtain large amounts of purified prothymosin α. Since human tissue culture cells contained insufficient amounts for use on a moderate scale, prothymosin α was purified to homogeneity from commercial, frozen calf thymus. The method used was a modification of our two-step procedure. In the first step, prothymosin α, alone among proteins, is recovered from the aqueous phase of a phenol extraction and, in the second, it is separated from

contaminating nucleic acids and carbohydrate either electrophoretically, or by HPLC. Bovine prothymosin α resembles the human protein with two exceptions—the Ser residue at position 83 of the human is replaced by an Ala residue, making ser 83 unlikely as a phosphorylation substrate, and Asp 31 of the human becomes a Glu in the bovine protein. The three relevant bovine peptides have now been isolated—a 57-mer bearing residue 83, a 9-mer bearing residues 8 and 9, and an NH_2-terminal peptide. Since the substitutions in the largest peptide are minor and the others are identical, the bovine system serves as model for the study of human prothymosin α.

The relationship between phosphorylation of prothymosin α and function has been addressed using synchronized cells. With a pulse-labeling technique, we have ascertained that prothymosin α is phosphorylated throughout the cell cycle. Since we have also measured the half-life of the protein in pulse-chase experiments with [^{3}H]glutamic acid and obtained a value of ~8 hours, it is unlikely that prothymosin α performs a cell cycle dependent function. However, it should be pointed out that we cannot yet separate phosphorylated from unphosphorylated prothymosin α, nor can we measure the specific activity of the phosphate in the radioactive protein. Hence, it is not clear whether prothymosin α is phosphorylated constitutively, or whether the two forms coexist and perform different functions.

Prothymosin α pre-mRNA is alternatively spliced. In view of the involvement of prothymosin α with the proliferative aspects of cellular metabolism, it became necessary to investigate the function and provenance of the two types of prothymosin α transcripts. Previously, we isolated a full length cDNA clone, which encodes a protein of 111 amino acids, from an SV40-transformed human fibroblast library; we also isolated partial clones derived from human lymphocytes. The latter encode a protein of 110 amino acids with the same sequence as that encountered in the fibroblast clone, but lacking the glutamic acid codon, GAG, responsible for the amino acid at position 39 of the mature protein. We have now shown that the two transcripts do not arise as a result of alleles at the same locus. We believe that alternative splicing occurred because the intronic sequence in question, $(Py)_6G(Py)_2GAG\downarrow\mathbf{GAG}\downarrow$, provided two closely spaced splice acceptor AG dinucleotides. If the splice were to occur at the first arrow, the GAG codon in boldface would form the first triplet of exon 3, producing the fibroblast type cDNA; splicing at the second arrow, a violation of the consensus rules for splice site selection, would sequester the triplet in the intron and generate the lymphocyte type of prothymosin α. Alternative splicing raised the possibility that the different forms of prothymosin α reflected one or more of the following: (1) the tissue in which the mRNA was processed; (2) viral or spontaneous transformation of normal cells; or (3) the conversion from a primary culture to a cell line. Our study has revealed that the smaller prothymosin α transcript prevails, with the larger representing about 10% of

prothymosin α transcripts regardless of the source. We do not know the phosphorylation status or functional relevance of the two forms. However, the violation of consensus splice site selection which produces the abundant form of prothymosin α suggests that a G residue in the -3 position of the splice acceptor site is unacceptable. Using a new cloning technique devised by us for making small substitutions in large genes, we are currently preparing several constructs designed to study the affects of GAG motifs at the ends of introns. We suspect that a single GAG will not be spliced, but that two or more can be accommodated, albeit aberrantly.

The six members of the human prothymosin α gene family have been cloned and sequenced. One gene contains introns, and appears to be the source of all isolated prothymosin α cDNAs. The remaining five genes are processed pseudogenes meeting, where applicable, the four defining criteria as follows: (1) they lack introns; (2) they differ from each other and from the functional gene immediately upstream and downstream of the transcribed region; (3) they contain terminal poly A encoded in the DNA; and (4) they exhibit short direct repeats flanking the transcribed region. The repeats are thought to occur when the pseudogenomic transcript is inserted into staggered breaks in genomic DNA. Four of the processed pseudogenes have consensus TATA elements upstream of sequences nearly identical to the transcriptional start region of the intron-containing gene. Those four genes also contain Kozak sequences upstream of the translational start, and open reading frames coding for proteins closely related to prothymosin α. In two of the pseudogenes, the encoded proteins differ from the product of the parental gene at only 2 and 4 locations, respectively. Thus, they, like mammalian prothymosins in general, are highly conserved. The fifth pseudogene encodes a different protein owing to an upstream translational initiation start site and multiple deletions and insertions. Because the potential for expression exists in this system, a search for pseudogenomic transcripts was undertaken using the polymerase chain reaction to amplify reverse transcripts of mRNAs from many human tissues and bulk DNA from several human cDNA libraries. Evidence for pseudogenomic transcripts was not obtained. Therefore, we conclude that the human prothymosin α gene family contains only one functional gene.

The function of prothymosin α is a major concern. Earlier, we observed that antisense-treated cells synthesized about 50% of the normal amount of RNA, under a rigorously defined set of conditions. Since prothymosin α is essential for cell division and is located exclusively in the nucleus, a DNA-related function seemed logical. Toward this end we have developed a model system for assessing DNA perturbations and found that low concentrations of prothymosin α, but not poly L or D glutamic acid, cause dramatic effects. Most recently, we have applied our findings to myeloma cells and once again we have obtained evidence for DNA-modifying activity. Tentatively, we have ruled out topoisomerase as an intermediary in these processes.

Publication:

Manrow RE, Leone A, Krug MS, Eschenfeldt WH, Berger SL. The human prothymosin α gene family contains several processed pseudogenes lacking deleterious lesions. Genomics 1992;13:319-31.

SUMMARY STATEMENT

LABORATORY OF MOLECULAR BIOLOGY

DCBDC, NCI

OCTOBER 1, 1991 to SEPTEMBER 30, 1992

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

Immunotoxin and Recombinant Toxin Therapy of Cancer:

To develop new cytotoxic agents for cancer treatment, I. Pastan, D. FitzGerald and colleagues have attached *Pseudomonas* exotoxin (PE) and genetically modified forms of PE to monoclonal antibodies (mAbs) or growth factors to create cell specific cytotoxic agents. A mutant form of PE, LysPE38, has been chemically attached to mAb B3. This immunotoxin, B3-LysPE38, causes regression of human tumors in mice with a large therapeutic window. Clinical grade mAb B3 has been prepared and clinical grade LysP38 is being prepared for an immunotoxin trial scheduled to begin in 1992. Other mutant forms of LysPE38 have been evaluated for the production of immunotoxins. One of these in which the lysine residues at the end domain III have been mutated makes a more active immunotoxin. A single chain immunotoxin (B3(Fv)-P38 KDEL) has been prepared which also causes tumor regression in mice. The conditions of producing B3(Fv)-P38 KDEL have been improved by studying refolding conditions and using mutant linker and connector molecules. Refolding can also be stimulated by the enzymes GroE and protein disulfide isomerase. The side chains of the amino acids in domain II have been changed to alanine residues to determine which side chains are important for the cytotoxic action of PE. Single domain immunotoxins have also been made with B3(Fv)-P38 KDEL and the results show that both the light and heavy chain interact with the B3 antigen. Indium labeled B3 has been prepared and shown to be a very effective imaging agent using human tumors growing in nude mice. Single chain recombinant immunotoxins have been made with antibodies that react with the erbB2 oncogene, with an antibody (C242) that binds with many human colon cancers and with antiTac that binds to the IL2 receptor present in many lymphomas and leukemias. These agents are in preclinical development. Other immunotoxins have been prepared that react with B cell lymphomas and the p75 subunit of the IL2 receptor. Recombinant toxins with longer half lifes have been created by inserting the C_H2 domain of human IgG1 between the ligand and the toxin domains.

Development of Immunotoxins for Cancer:

D. FitzGerald and colleagues have studied how PE is processed to an active C-terminal fragment which translocates to the cell cytoplasm and inhibits protein synthesis by ADP-ribosylating EF-2. At the cell surface, PE binds to a large MW glycoprotein which has been identified as the α_2-macroglobulin receptor (α_2M-R). A 39 kD receptor associated protein (RAP) blocked PE binding to cells and pretreatment of cells with RAP prevented toxin-mediated inhibition of protein synthesis. Once delivered to the endosomal compartment, PE is cleaved by an membrane-associated protease (MAP). To prepare the protease, crude membranes are treated with papain to release the MAP in a soluble form.

In the endosome PE is cleaved between arginine 279 and glycine 280 and then reduced to generate the 37 kD C-terminal fragment. In the presence of excess PE553D (a mutant form of PE lacking ADP-ribosylating activity), the toxicity of native PE is greatly reduced. Excess PE553D does not compete strongly for the binding of native PE to its surface receptor, rather it competes within cells for a step taken by the 37 kD fragment *en route* to the cytosol. Mutant forms of PE553D with substitutions for trp 281 compete poorly. PE553D W281A competes 100-fold less well than PE553D despite the fact that PE553DAla281 binds to cells, is internalized, and is cleaved appropriately by the cellular protease. In PE, glycine 280 can be changed to methionine without loss of biological activity. This allows the production of recombinant forms of PE that begin at residue 280 (with methionine in this position) and do not need to be processed within cells.

Monoclonal Antibodies to Cancer Cells:

K. Chang has isolated MAb K1 which reacts with many non mucinous ovarian cancers, mesotheliomas and some squamous cell carcinomas. The antigen with which MAb K1 reacts is a 40,000 molecular weight glycoprotein containing about 25% carbohydrate and is attached to the cell membrane through a phosphatidylinositol linkage. The antigen is present in normal mesothelium as well as many cancers. Recently, cDNAs encoding the K1 antigen have been isolated and are currently being sequenced. The K1 antigen is poorly internalized so that K1-PE40 is not very cytotoxic to target cells. The cDNAs encoding the light and heavy chains of K1 were isolated and a single chain immunotoxin constructed. In addition, Fab fragments have been made in *E. coli* and will be used for imaging studies.

Mechanisms of Thyroid Hormone Action in Animal Cells

To understand the molecular mechanism(s) by which the thyroid hormone, 3,3',5-triiodo-L-thyronine (T_3) promotes growth and differentiation, S.-y. Cheng and colleagues studied the structure and activity of two cellular thyroid hormone binding proteins. h-TRβ1 was found to be a phosphoprotein *in vivo* with Ser, Thr and Tyr as the phosphorylation sites in the ratio of 85:10:5. Okadaic acid, a potent inhibitor of phosphatase 1 and 2A, stimulated the phosphorylation of h-TBβ1. The increase in phosphorylation was accompanied by an increase in receptor-mediated transcription. h-TRβ1 purified from *E. coli* was phosphorylated *in vitro* by the endogenous kinase from cellular extracts. Ser, Thr and Tyr were phosphorylated in a similar ratio to that found *in vivo*. The *in vitro* phosphorylation was stimulated by okadaic acid. Phosphorylation did not affect the binding of h-TRβ1 to T_3. However, phosphorylation of h-TRβ1 resulted in an increase of its binding to DNA and conferred on it the ability to bind to nuclear accessory proteins. These results indicate that phosphorylation plays an important role in the transcriptional activity of h-TRβ1.

The transcriptional activity of h-TRβ1 is T_3-dependent. To explore the possible role of p58-M_2 as a regulator for T_3 action, they evaluated the effect of the availability of cytoplasmic T_3 on the modulation of transcriptional responses of T_3 receptor. In human choriocarcinoma JEG-3 and monkey COS-1 cells, p58-M_2 is a monomer of the tetrameric pyruvate kinase, subtype M_2, called PKM_2, which does not bind T_3. The *in vivo* monomer-tetramer interconversion is regulated by glucose via fructose 1,6-bisphosphate. At the physiological T_3 concentration, lowering the glucose concentration led to an increase in the cellular concentration of p58-M_2 and a concomitant reduction in the transcriptional activity of a transfected h-TRβ1 in both cell lines. In the absence of glucose, the transcriptional activity of h-TRβ1 in JEG-3 and COS-1 cells was reduced by 65-75% and 90-95%, respectively. These findings demonstrate an important prenuclear step in the modulation of the gene regulating activity of the T_3 receptors.

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

G. Merlino and colleagues use transgenic technology in which foreign DNA is stably introduced into the mammalian germ line to investigate the role of growth factors, receptors and oncogenes in the initiation and development of neoplasia, and to establish useful animal models to study the pathogenesis of human disease.

To gain information about the role of TGFα in human diseases, transgenic mice were made bearing the human TGFα gene. TGFα overexpression was found to induce hepatocellular carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and a hypertrophic gastropathy resembling Menetrier's disease. Detailed molecular analysis of transgenic liver neoplasia has shown that TGFα promotes tumor formation and plays a role in tumor progression. Furthermore, other factors that may collaborate in TGFα-induced hepatocarcinogenesis include expression of *c-myc*, insulin-like growth factor II, sex hormones, and the genetic background upon which the transgene operates.

Merlino and colleagues have generated transgenic mice containing foreign DNA encoding other interesting growth and differentiation factors. Transgenic mice made using an activated form of an EGF-related gene, *int-3*, develop severe hyperplastic lesions of secretory glands and neoplasia of the salivary and mammary glands. The latter were also arrested in development and lactation deficient in all female *int-3* transgenic mice. Male mice were sterile due to epididymal hyperplasia. These findings demonstrate that expression of the activated *Notch*-related *int-3* gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia.

Regulation of Gene Activity:

I. Pastan, A. Johnson and colleagues have studied factors controlling the expression of the EGF receptor (EGFR) gene. They have previously isolated and characterized the promoter region of the gene and identified transcription factors that act as positive or negative regulators for this promoter region. A cDNA clone encoding a negative regulator, GCF, was isolated and the transcription of GCF examined in clonal cell lines.

GCF antisera reacted with a 97 kilodalton protein in cell extracts. GCF expression was also examined in EGFR negative and EGFR positive cell lines and found to be higher in EGFR negative cell lines. Cell fractionation studies show that GCF is primarily located in the nucleus but that some forms appear in the cytoplasm. GCF was determined to be a phosphoprotein with a 6-8 hour half life.. Another cDNA clone has been isolated that hybridizes to one of these RNA species that contains homology to the GCF cDNA.

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

S. Adhya and colleagues have shown that each of the two promoters of the *gal* operon of *E. coli* is negatively regulated by two repressors: the classical Gal repressor and the newly discovered Gal isorepressor. Both act by binding to the same two spatially separated operators, O_E an O_I. Gal repressor and isorepressor belong to a family of bacterial regulatory proteins, we termed GalR family. An alignment of the proteins of the family show 60% homology throughout the entire sequences. They have established that both repressor and isorepressor participate in the modulation of a *gal* regulon, which includes, besides the *gal* operon, the *mgl* operon encoding one of the galactose transport systems and *galR* and *galS*, the genes for Gal repressor and isorepressor respectively. The isorepressor

has a major and the repressor a minor role on the negative control of the *mgl* operon. The latter carries a *gal* operator element at the -60 region. The effect of isorepressor on *mgl* is at the level of transcription initiation.

S. Adhya and H. Choy have developed an *in vitro* transcription assay to study regulation with purified proteins. It uses supercoiled DNA minicircles which carry only the *gal* promoter segment. In this system, using DNA minicircle containing the *gal* promoters with *lac* operator sequences at O_E and O_I, we have demonstrated the requirement of DNA looping *in vitro*. Lac repressor, which is capable of association into a tetramer and forming a DNA loop as observed by electron microscopy, repressed transcription from *P*1 and *P*2 while a nonlooping Lac repressor mutant failed to show normal repression from both promoters. Repression mediated by DNA looping inhibited the synthesis of complete as well as abortive transcripts, demonstrating that the repression was on the formation or activity of the initial transcribing complex. DNase foot printing results indicated that repressor does not inhibit RNA polymerase binding.

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

The cyclic AMP receptor protein (CRP) of *Escherichia coli* is a DNA binding protein. S. Garges and S. Adhya are studying a number of aspects of the mechanism of action of CRP. To study the cAMP-induced allosteric change, they have isolated allosteric-defective *crp* mutants and intragenic suppressors of that class. They conclude from *in vivo* and *in vitro* work with these mutant CRPs that the hinge of CRP and the part of the protein immediately adjacent to the DNA binding region are important in the cAMP-induced allosteric change. They have found that CRP binding can cause a B to A-form transition in DNA; this has implications in how CRP activates transcription. It appears that CRP induces a change in the DNA that might be transmitted to the promoter. By introducing changes in the DNA between where CRP binds and the promoter, they can affect CRP action: a nick or single-base gap hardly perturbs activation, a two-base gap has lower activity, and a four-base gap is non-functional.

They have also found that the *pts* operon which encodes proteins necessary for certain sugar uptake is regulated in a novel way by CRP. There are two CRP binding sites and there may be interaction between CRPs bound at the two sites.

Transcription Mechanisms and Structure-Function Analysis of RNA Polymerase of *E. coli*:

D. Jin is studying the catalytic center and the regulatory domain(s) of RNA polymerase of *Escherichia coli*. The effect of mutations in RNA polymerase that confer rifampicin resistance (rif^r) on different steps of transcription have been analyzed. RNA polymerases with altered rifampicin binding are likely to be also altered in some vital transcription processes. By studying the correlation between a particular rif^r RNA polymerase and its altered transcription property, one can assign the functional role of the rifampicin binding site(s) of RNA polymerase. Some of rif^r RNA polymerases are defective in promoter clearance (a transition step in transcription at which an RNA polymerase exits from the initiation stage to the elongation stage). These polymerases also enhance abortive initiation products. The mechanisms underlying the defect in promoter clearance of one mutant polymerase in which there is an Arg 529 Cys change in the β subunit have been studied in detail. This mutant RNA polymerase has reduced affinity for nucleoside triphosphates during initiation and is subject to a high Km barrier during the promoter clearance process. Since this rif^r RNA polymerase also has reduced affinity for the same nucleoside

triphosphates during elongation, it is plausible that the amino acid residue Arg 529 of the β subunit is part of the catalytic center of RNA polymerase.

DNA Replication *in vitro*:

S. Wickner and D. Skowyra have been interested in the mechanism of action of chaperone proteins in protein folding. They have been using an *in vitro* replication system that replicates DNA carrying the plasmid P1 origin of replication as a model system to study the function of three *E. coli* heat shock proteins, DnaJ, DnaK (the Hsp70 homolog), and GrpE. They found that DnaJ and DnaK, in an ATP-dependent reaction, activate the sequence specific DNA binding of the P1 initiator protein, RepA. They discovered that activation converts RepA dimers to monomers and that the monomer form binds with high affinity to the P1 origin DNA. The enzymatic activation can be mimicked by converting RepA dimers to monomers by urea denaturation followed by renaturation. They also found that urea-treated RepA bypasses the requirement for DnaJ, DnaK, and GrpE in *in vitro* complementation assays with crude extracts of *dnaJ, dnaK,* and *grpE* mutant cells without the addition of purified DnaJ, DnaK, or GrpE, respectively. Thus, DnaJ, DnaK, and GrpE are required exclusively for the activation of RepA for P1 plasmid DNA synthesis *in vitro*.

Bacterial Functions Involved in Cell Growth Control:

S. Gottesman and colleagues have been studying the role that protein degradation plays in regulating gene expression and have continued with studies on the linkages between chromosome synthesis and partition of chromosomes during cell division. As part of continuing studies on the regulation of capsular polysaccharide synthesis by the unstable regulator RcsA, they have found that a downstream trans-acting regulator of *rcsA* synthesis encodes RNAs which apparently mediate the regulatory effect. Mutations in both the regulator and target will allow further characterization of the mode of regulation. Mutations in the putative phosphorylation site of RcsB support a role for phosphorylation in capsule regulation. They are investigating the role of the heat shock chaperone proteins in degradation of substrates of the Lon ATP-dependent protease and initiating studies on the recognition specificity of Lon. Studies on the role and regulation of the Clp energy-dependent protease have concentrated on developing strains for the tight regulation of Clp synthesis, and investigating the function of a newly discovered alternate regulatory subunit, ClpX.

Molecular Modeling:

The main research interest of B. K. Lee and colleagues is to study by various theoretical means the forces that govern the structure and interaction of globular protein molecules, to predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties. Following were accomplished: (1) The molecular graphics modeling program, GEMM, has been improved; (2) The origin of the hydrophobic effect has been investigated and its magnitude has been estimated relative to the non-hydrophobic effect on the stability of globular proteins; (3) Two different approaches have been devised to predicting the three-dimensional structure of a globular protein from its amino acid sequence alone and the strength and the shortcomings of each approach have been evaluated.

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells:

In collaboration with M. M. Gottesman, Laboratory of Cell Biology, the role of drug resistance in human cancer has been studied. Resistance to chemotherapy of human cancers has been studied by establishing *in vitro* and *in vivo* genetic systems which mimic development of drug resistance. A major mechanism of multidrug resistance in human cancer is expression of the *MDR*1 gene which we have cloned and sequenced. Expression of the *MDR*1 gene carried by retroviral vectors can be used to transduce cells to multidrug resistance and has been used to confer resistance to mouse bone marrow cells *in vitro* and *in vivo*. Mechanistic studies on P-glycoprotein suggest that it is involved in removing drug directly from the plasma membrane. To study mechanism, we have generated many mutants and initiated biochemical studies including the purification of P-glycoprotein and reconstruction of drug-dependent ATPase activity in artificial liposomes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08000-22 LMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

PI:	A. Johnson	Staff Fellow	LMB, NCI
	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
Other:	A. Reed	Howard Hughes Fellow	LMB, NCI
	H. Yamazaki	Visiting Fellow	LMB, NCI
	L. Beguinot	Visiting Associate	LMB, NCI

COOPERATING UNITS *(if any)*
N. C. Popescu, Laboratory of Biology, DCE, NCI, NIH

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Biology

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-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.)*

Overexpression of the EGF receptor (EGFR) gene has been detected in many human tumors. In addition, cells in culture can be transformed by the expression of high EGFR levels. To study the regulation of the EGFR gene, we have previously isolated and characterized the promoter region of the gene and identified transcription factors that act as positive or negative regulators for this promoter region. A cDNA clone encoding a negative regulator, GCF, was isolated and the transcription of GCF examined in clonal cell lines. GCF antisera reacted with a 97 kilodalton protein in cell extracts. GCF expression was also examined in EGFR negative and EGFR positive cell lines and found to be higher in EGFR negative cell lines. Cell fractionation studies were performed to show that GCF was primarily located in the nucleus but that some forms appear in the cytoplasm. GCF was determined to be a phosphoprotein with a 6-8 hour half life.. Another cDNA clone has been isolated that hybridizes to one of these RNA species that contains homology to the GCF cDNA.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-539

Major Findings:

The EGFR promoter binds transcription factors that have been shown to be either positive or negative regulators. GCF is a transcription factor that has been shown to repress transcription of the EGFR promoter. We have now examined the expression of GCF in EGFR negative and EGFR positive cell lines. Antisera to GCF was generated using a *Pseudomonas* exotoxin GCF fusion protein. The antisera reacted positively to *in vitro* made GCF and recognized a 97 kilodalton protein in cell extracts. EGFR negative cell lines, HUT102 and U266, were found to express GCF. EGFR expressing cell lines, KB, A431 and AGS, were also found to express GCF, but in lower amounts. Cell fractionation studies showed that GCF is predominantly localized in the nucleus. GCF was found to be a phosphoprotein and the phosphorylated from was primarily associated with the nucleus. Phosphorylation of GCF was stimulated by okadaic acid, PMA and cAMP. GCF has a half-life of 6-8 hours in HUT102 cells.

GCF is encoded by a 3 kilobase mRNA. The GCF cDNA hybridizes to additional RNA species of 4.5 and 1.2 kilobases. We have isolated and sequenced a cDNA clone that hybridizes to the 4.5 kilobase RNA species. This cDNA contains a 274 nucleotide stretch of 100% identity to GCF. The homology includes the 5' untranslated region of GCF and a portion of the 5' coding region.

We have generated a population of clones that contain a stably integrated GCF-specific homologous recombination vector. These clones were doubly selected with G418 and gancyclovir after transfection with the homologous recombination vector containing a portion of the GCF gene, the neomycin resistance and thymidine kinase gene. These clones will be analyzed by Southern blot hybridization analysis for those in which the GCF locus has been disrupted.

Publications:

Johnson AC, Kageyama R, Popescu N, Pastan I. Expression and chromosomal localization of the human transcriptional repressor, GCF, J Biol Chem 1992;267:1689-94.

Zheng ZS, Polakowska R, Johnson A, Goldsmith LA. Transcriptional control of EGFR by retionic acid, Cell Growth Differ 1992;3:225-32.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08010-19 LMB

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Monoclonal Antibodies to Cancer Cells

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

PI:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
Others:	K. Chang	Visiting Associate	LMB, NCI
	A. Rutherford	Biologist	LMB, NCI

COOPERATING UNITS *(if any)*

H. Pass, H. W. Pogrebniak, Surgery Branch, NCI, NIH; M.-S. Tsao, Dept. Pathology, Montreal General Hospital, Montreal, Quebec, Canada

LAB/BRANCH: Laboratory of Molecular Biology

SECTION: Ultrastructural Cytochemistry Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

K. Chang has isolated MAb K1 which reacts with many non mucinous ovarian cancers, mesotheliomas and some squamous cell carcinomas. The antigen with which MAb K1 reacts is a 40,000 molecular weight glycoprotein containing about 25% carbohydrate and is attached to the cell membrane through a phosphotidylinositol linkage. The antigen is present in normal mesothelium as well as many cancers. Recently, cDNAs encoding the K1 antigen have been isolated and are currently being sequenced. The K1 antigen is poorly internalized so that K1-PE40 is not very cytotoxic to target cells. The cDNAs encoding the light and heavy chains of K1 were isolated and a simgle chain immunotoxin constructed. In addition, Fab fragments have been made in *E. coli* and will be used for imaging studies.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

Publications:

Chang K, Pai LH, Batra JK, Pastan I, Willingham MC. Characterization of the antigen (CAK1) recognized by monoclonal antibody K1 that is present on ovarian cancers and normal mesothelium, Cancer Res 1992;52:181-6.

Chang K, Pastan I, Willingham MC. Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium, International J Cancer 1992;50:373-81.

Chang K, Pai LH, Pass H, Pogrebniak HW, Tsao M-S, Pastan I, Willingham MC. Monoclonal antibody K1 reacts with epithelial mesothelioma but not lung adneocarcinoma, Amer J Surgical Pathol 1992;16:259-68.

Chang K, Pastan I, Willingham MC. Frequent expression of the tumor antigen CAK1 in squamous cell carcinomas, Int J, Cancer 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08710-15 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

DNA Replication *in vitro*

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

PI:	S. Wickner	Research Chemist	LMB,	NCI
Other:	D. Skowyra	Visiting Fellow	LMB,	NCI

COOPERATING UNITS *(if any)*

Keith McKenney and Joel Hoskins, Center for Advanced Research in Biotechnology, Gaithersburg, MD 20850

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	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 have been interested in the mechanism of action of chaperone proteins in protein folding. We have been using an *in vitro* replication system that replicates DNA carrying the plasmid P1 origin of replication as a model system to study the function of three *E. coli* heat shock proteins, DnaJ, DnaK (the Hsp70 homolog), and GrpE. We found that DnaJ and DnaK, in an ATP-dependent reaction, activate the sequence specific DNA binding of the P1 initiator protein, RepA. We discovered that activation converts RepA dimers to monomers and that the monomer form binds with high affinity to the P1 origin DNA. The enzymatic activation can be mimicked by converting RepA dimers to monomers by urea denaturation followed by renaturation. We found that urea-treated RepA bypasses the requirement for DnaJ, DnaK, and GrpE in *in vitro* complementation assays with crude extracts of *dnaJ, dnaK,* and *grpE* mutant cells without the addition of purified DnaJ, DnaK, or GrpE, respectively. Thus, DnaJ, DnaK, and GrpE are required exclusively for the activation of RepA for P1 plasmid DNA synthesis *in vitro*.

GPO 914-918

Major findings:

DnaK is a major heat shock protein of *E. coli* and the homolog of eucaryotic hsp70. Heat shock proteins protect cells against the stress of heat shock and repair damage done by heat. DnaK, in addition to participating in the heat shock response, is essential for normal growth in non-stress conditions. It functions with two other heat shock proteins, DnaJ and GrpE, in the DNA replication of phage λ and several plasmids, including P1. We have been studying the mechanism of action of three heat shock proteins in the initiation of DNA replication of plasmids carrying the P1 origin, *ori*P1.

We found that DnaJ and DnaK activate the sequence specific DNA binding of the P1 initiator protein, RepA. RepA is a dimer in solution and forms a stable complex with DnaJ, containing a dimer each of RepA and DnaJ. DnaK, in an ATP-dependent reaction stimulates the specific DNA binding activity of RepA by about 100-fold. This reaction does not require DNA. We discovered that activation converts RepA dimers to monomers and that the monomer form binds with high affinity to *ori*P1 DNA. The enzymatic activation can be mimicked by converting RepA dimers to monomers by urea denaturation followed by renaturation.

We proposed that in normal growth conditions, native proteins are identified as targets for DnaK via a protein tag. In our system, DnaJ is the protein specific tag. The RepA-DnaJ tetramer targets RepA for DnaK action. DnaK recognizes the RepA-DnaJ complex and either RepA or DnaJ acts as an allosteric effector of the DnaK ATPase activity. This produces a conformational change in DnaK which releases active, monomeric RepA protein.

We investigated whether the DnaJ and DnaK catalyzed activation of RepA DNA binding was required for *ori*P1 DNA replication *in vitro* and whether GrpE was also involved at this step. We used the RepA protein encoded by P7, a plasmid prophage closely related to P1, because it is more active than P1 RepA in catalyzing *ori*P1 DNA synthesis in *in vitro* replication reactions. P7 RepA is identical to P1 RepA except for a single amino acid substitution. The DNA binding activity of P7 RepA, like P1 RepA, could be stimulated by incubation with DnaJ, DnaK and ATP or by treatment with urea followed by dialysis to remove the denaturant. P7 RepA, like P1 RepA, was a dimer in solution and urea-treatment simultaneously converted P7 RepA into the monomer form and activated origin DNA binding.

We showed by *in vitro* complementation assays that replication of *ori*P1 DNA initiated by P7 RepA requires DnaJ, DnaK and GrpE. We then asked whether these three proteins function in *ori*P1 DNA replication solely to activate RepA. The alternatives are: (i) DnaJ and DnaK are required to activate RepA and in addition are required at a subsequent stage of DNA replication. (ii) RepA monomerization is required for *ori*P1 DNA replication but can be carried out by other proteins in the crude extracts. The heat shock proteins would then be required at another stage, as they are in *ori*λ DNA replication. (iii) RepA monomerization is not essential and the heat shock proteins are required at another step in *ori*P1 DNA replication.

If DnaJ, DnaK, and GrpE were needed solely for activation of P7 RepA, then urea-activated RepA should catalyze *ori*P1 DNA synthesis in complementation assays with *dnaJ*, *dnaK*, and *grpE* mutant extracts without the addition of purified DnaJ, DnaK, or GrpE, respectively. We found this to be the case. We obtained similar results with P1 RepA, however, DNA synthesis was lower due to the lower specific activity of P1 RepA protein compared to P7 RepA. Thus, DnaJ, DnaK, and GrpE are required exclusively for the activation of RepA for *ori*P1 DNA synthesis *in vitro*. Although with our standard reaction conditions, GrpE is not required for the *in vitro* activation of RepA, we have discovered that it is required with other reaction conditions.

Therefore, the essential role of these three heat shock proteins in this replication system is to change the quaternary structure of a single protein, RepA.

Publications:

Wickner S, Hoskins J, McKenney K. DnaJ and DnaK heat shock proteins activate sequence specific DNA binding by RepA. In: Lundquist S, Maresca B, eds. Heat shock. Berlin: Springer-Verlag, 1992;67-76.

Wickner S, Hoskins J, McKenney K. The replication initiator protein of P1 is activated by two E. coli heat shock proteins, DnaJ and DnaK. In: Hughes P, Kohiyama M. eds. DNA replication: Regulatory mechanisms. Berlin: Springer-Verlag, 1992;347-58.

Wickner S, Hoskins J, McKenney K. Monomerization of RepA dimers by heat shock proteins activates binding to DNA replication origin, Proc Natl Acad Sci USA 1991;88:7903-7.

Baker T, Wickner S. DNA replication in *E. coli,* Ann Rev Genetics 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08714-15 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Bacterial Functions Involved in Cell Growth Control

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

PI:	S. Gottesman	Chief, Biochemical Genetics Section	LMB,	NCI
Other:	W. Clark	Chemist	LMB,	NCI
	N. Trun	Staff Fellow	LMB,	NCI
	V. de Crecy Lagard	Visiting Fellow	LMB,	NCI
	D. Sledjeski	IRTA Fellow	LMB,	NCI
	Y. Jubete	Visiting Fellow	LMB,	NCI
	J. Cha	Guest Researcher	LMB,	NCI

COOPERATING UNITS *(if any)*

M. Maurizi, Laboratory of Cell Biology, NCI; J. Trempy, Dept. of Microbiology, Oregon State U.; D. Gutnick, Tel Aviv U.; V. Stout, Arizona State U.; A. Lobner-Olesen, The Technical University of Denmark; Tom Patterson, DuPont; M. Zylicz, University of Gdansk

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	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 been studying the role that protein degradation plays in regulating gene expression and have continued with studies on the linkages between chromosome synthesis and partition of chromosomes during cell division. As part of continuing studies on the regulation of capsular polysaccharide synthesis by the unstable regulator RcsA, we have found that a downstream trans-acting regulator of *rcsA* synthesis encodes RNAs which apparently mediate the regulatory effect. Mutations in both the regulator and target will allow further characterization of the mode of regulation. Mutations in the putative phosphorylation site of RcsB support a role for phosphorylation in capsule regulation. We are investigating the role of the heat shock chaperone proteins in degradation of substrates of the Lon ATP-dependent protease and initiating studies on the recognition specificity of Lon. Studies on the role and regulation of the Clp energy-dependent protease have concentrated on developing strains for the tight regulation of Clp synthesis, and investigating the function of a newly discovered alternate regulatory subunit, ClpX.

GPO 914-916

Major Findings:

1. Lon:

A variety of observations suggest that Lon recognition of substrates may be somewhat different for degradation of naturally unstable proteins such as RcsA, SulA, and lambda N protein, than for the large number of abnormal proteins which become subject to Lon degradation. We have predicted that the heat shock chaperone proteins such as DnaJ and DnaK may be involved in presentation of abnormal proteins for degradation by Lon, but may not play a role in degradation of the naturally unstable proteins. This is being tested directly, examining the turnover of both abnormal proteins and RcsA, SulA, and N, in isogenic strains, comparing those carrying mutations in the heat shock gene *dnaJ* with strains wild type for heat shock proteins. A understanding of the role of these heat shock proteins in Lon-dependent turnover will be essential for the broader examination of Lon target specificity.

2. Capsule synthesis:

We have continued our analysis of the regulation of capsular polysaccharide synthesis. Our model for capsule synthesis suggests that there are two pathways for activating transcription of the target *cps* genes: 1) Activation of the membrane sensor, RcsC ,which is probably a protein kinase (by analogy to homologous sensors in other systems). Activated RcsC would lead in turn to the activation (phosphorylation) of the positive regulator RcsB. 2) The accumulation of the unstable positive regulator RcsA. We predict that RcsA can interact with RcsB to increase *cps* transcription even in the absence of RcsB phosphorylation. Activation of this pathway is limited in wild-type cells by the extremely low level of RcsA accumulation. In cells mutant in the Lon ATP-dependent pathway, RcsA is stable, and therefore RcsA accumulation increases significantly, and capsule synthesis is also increased.

A direct prediction of this model is that it should be possible to isolate mutations in *rcsB* which block activation by RcsC, but can still interact with RcsA to stimulate capsule synthesis. We have isolated site-directed changes in the conserved aspartate which is expected to serve as the phosphorylation site for RcsB, necessary for RcsC activation. The effect of these changes is being examined in collaboration with Valarie Stout. On plasmids or as single copy recombinants to the chromosome, a change of the aspartate to an asparagine inactivates RcsB for capsule synthesis by all pathways tested. A change of the aspartate to glutamate, when present on an *rcsB* plasmid, allows RcsB activity in the RcsA dependent pathway, and blocks the lethality of *rcsB*$^+$ plasmids in *rcsC* mutant hosts. We are currently transferring this mutation to the chromosome for further tests. These results thus far suggest that the expected site of phosphorylation is critical for RcsB activity, but that phosphorylation, *per se*, (assuming the glutamate will not be phosphorylated) may not be necessary for activity.

Cells carrying single copies of an *rcsA-lacZ* transcription fusion carrying 1 kb of DNA upstream of the *rcsA* translational start have been constructed to examine the regulation of RcsA synthesis. LacZ is expressed at very low levels in these strains. Expression increases in the presence of a multi-copy plasmid carrying a fragment of DNA from the region downstream of *rcsA*. Protection experiments indicate that this downstream region encodes RNAs which apparently arise by processing of a single abundant transcript; mutations which block the effect of the downstream region on *rcsA* transcription change the processing pattern of these RNAs. Mutations in the *rcsA* promoter have also been isolated which are resistant to the effects of the downstream region. Analysis of both sets of mutations should help in understanding the mechanism of regulation of *rcsA*.

3. Clp:

The Clp energy-dependent protease has two subunits. ClpA, an 83,000 MW protein, has two consensus nucleotide binding sites, and shares extensive homology with a family of proteins found in both prokaryotes and eukaryotes. A second member of this family, ClpB, is also present in *E. coli*. ClpP, a 23,000 MW protein, carries the active site serine for the protease, and is processed in a *clpP*-dependent proteolytic cleavage of the N-terminal 14 amino acids.

Recent work done by M. Zylicz in Poland suggests that the ClpP proteolytic subunit can combine with subunits other than ClpA to carry out energy-dependent protein degradation. In collaboration with him, we have found that one of these alternative subunits is identical to ClpX, the predicted protein product for a gene we had previously identified and sequenced. This gene appears to be part of the *clpP* operon, and lies between *clpP* and *lon* on the *E. coli* map. We are making deletion-insertion mutations in *clpX* to cross into the *E. coli* chromosome and determine the phenotype of *clpX* mutations with or without mutations in *clpA* or *clpB*.

The identification of ClpX as an alternative subunit for energy-dependent proteolysis emphasizes the importance of using mutations in *clpP* to analyze the *in vivo* role of the Clp proteases. We have observed that mutations in both *lon* and *clpP* lead to very poor growth and lethality at high temperatures in a number of strain backgrounds. The basis for this lethality is being examined. In addition, we are beginning a large scale screen for insertion mutations which are lethal in a *clpP* background, but viable in the presence of *clpP*$^+$, or show the reverse pattern. Since the insertions are made with *lac* fusion vectors, we can simultaneously screen for regulation of *lac* expression in a *clp*-dependent fashion. These experiments require a well-regulated source of ClpP. We have found that a low-copy plasmid carrying *clp*P under lambda pL regulation satisfies our requirements. We anticipate that any identified insertions can be tested for the behavior in *clpA*, *clpX*, *clpB*, or other yet to be identified mutations in regulatory subunits for ClpP.
Studies on the regulation of ClpA have suggested the possibility of an upstream site which helps stimulate high level expression of ClpA. Further deletion analysis of this region is being undertaken.

In collaboration with M. Maurizi (LCB, NCI), we have continued generating mutations at the cleavage site of *clpP*, in an attempt to answer two questions: 1) What does Clp recognize about this site, and 2) Is processing necessary for Clp activity? Thus far, we have not obtained any mutations which abolish Clp processing but retain activity. Many mutations have no effect on either activity or processing, while some mutations close to the cleavage site lead to reduced activity and abnormal processing (a larger intermediate accumulates). These results may suggest that recognition of the ClpP precursor may reside elsewhere than at the cleavage site *per se*.

4. Alp:

We have defined an activity which suppresses the loss of the Lon protease and leads to increased degradation of the SulA protein in an energy-dependent manner. The activity is dependent on the presence of the regulatory gene, *alp*, on a multi-copy plasmid. We had previously shown that *alp* acts to up-regulate a linked chromosomal gene, *slp*, which leads to excision of a previously unknown P4-like defective prophage. Excision of the prophage is necessary but not sufficient for expression of the new proteolytic activity. In addition to excision, the presence of a second multi-copy function, called for the moment *alpB*, seems to be necessary for full suppression of *lon* mutants. The *alpB* function is being defined by subcloning and mutagenesis, but appears to be contained within a kanamycin resistance transposon we used for mutagenesis of the original

plasmid. Excision of the cryptic prophage is recessive, suggesting that a function lost on excision interferes with expression of activity of the new protease. The negative regulator has been mapped to a bacterial gene whose 3' end crosses the prophage boundary and is, therefore, altered by excision of the cryptic prophage. This gene, *ssrA*, identified, mapped, and sequenced by D. Apirion and his coworkers, encodes a small stable RNA of unknown function.

We have begun the isolation and characterization of mutations which may define the site of action of the *ssrA* RNA, and presumably the gene for the induced protease. The first set of mutations isolated included a large cluster which proved to be in a previously identified gene which can be mutated to give a lower copy number for pBR322 and PACYC184-based vectors. These mutations confirm the necessity for a high copy kanamycin resistance gene to see full protease expression. Some of the mutations isolated do not appear to be of this class, and remain candidates for a site of RNA action. These mutations will be mapped and characterized further.

5. Cell Cycle Mutations:

We have selected *E. coli* mutants which have increased chromosome number as a way of identifying genes involved in chromosome partitioning. The mutants, called *mbr*, were selected as resistant to camphor, and map to four sites. In collaboration with A. Lobner-Olesen, the genome complement and size of individual cells under permissive and non-permissive conditions has been determined by flow cytometry. In combination with genetic analysis of epistasis, the results suggest that one of the newly identified genes, *mbrB*, may be involved in initiation of DNA replication, *mbrC* and *mbrD* (which appears to be an allele of *rpoB*, a subunit for RNA polymerase) are defective in the segregation of chromosomes at the end of the replication cycle, and the behavior of *mbrA* suggests that it defines a new linkage between replication and cell cycle.

Attempts to clone the *mbr* genes by standard techniques have been unsuccessful; the mutations are dominant, but appear not to be well tolerated on most plasmids, including those with a low copy number. In a diploid cell, insertions into the mutant (dominant allele) *mbrA* gene has allowed physical mapping of the region around the gene, and will now allow cloning of the disrupted gene. Similar experiments with *mbrC* are being pursued as well.

Second site suppressors of one of the *mbr* mutations, *mbrC*17, have been identified, and are being mapped. Further analysis of these suppressors should help to identify other components of the cell cycle machinery which interact with *mbrC*.

Publications:

Trun NJ, Gottesman S. Characterization of *Escherichia coli* mutants with altered ploidy, Res Microbiol 1991;142:195-200.

Stout V, Gottesman S. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12, Mol Microbiol 1991;5:1599-1606.

Trun NJ, Gottesman S, Lobner-Olesen A. Analysis of *Escherichia coli* mutants with altered DNA content, Cold Spring Harbor Symp Quant Biol 1991;56:353-58.

Parker CT, Kloser AW, Schnaitman CA, Stein MA, Gottesman S, Gibson BW. Role of the *rfaG* and *rfaP* genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12, J Bact 1992;174:2525-38.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08750-12 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Genetic Regulatory Mechanisms in *Escherichia coli* and Its Bacteriophage

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

PI:	S. Garges	Microbiologist	LMB, NCI
	S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
Others:	S. Ryu	Visiting Fellow	LMB, NCI
	V. Ivanov	Visiting Scientist	LMB, NCI
	N. Ogihara	Microbiologist	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Developmental Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.25	2.75	0.5

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The cyclic AMP receptor protein (CRP) of *Escherichia coli* is a DNA binding protein that can activate transcription when bound to specific sites near promoters. We are studying a number of aspects of the mechanism and consequences of action of CRP on transcription. To study the cAMP-induced allosteric change, we have isolated allosteric-defective *crp* mutants and intragenic suppressors of that class. We conclude from *in vivo* and *in vitro* work with these mutant CRPs that the hinge of CRP and the part of the protein immediately adjacent to the DNA binding region are important in the cAMP-induced allosteric change. We have found that under certain conditions, CRP binding can cause a B to A-form transition in DNA. This could have implications in determining how CRP activates transcription. From our work, it appears that CRP induces a change in the DNA that might be transmitted to the promoter. By introducing changes in the DNA between where CRP binds and the promoter, we can affect CRP action: a nick or single-base gap hardly perturbs activation, a two-base gap has lower activity, and a four-base gap is non-functional.

We have found that the *pts* operon which encodes proteins necessary for certain sugar uptake is regulated in a novel way by CRP. There are two CRP binding sites and there may be interaction between CRPs bound at the two sites.

Major Findings:

1. How CRP activates transcription:

We have continued our work on how the cyclic AMP (cAMP) receptor protein (CRP) activates transcription at different promoters in *Escherichia coli*. There are several steps in the pathway to activation by CRP: (i) the cAMP-induced conformational change in CRP necessary for DNA binding (ii) the binding of CRP to DNA and (iii) CRP of transcription initiation.

a. The cAMP-induced conformational change:

Previously we identified the hinge region connecting the cAMP-binding domain to the DNA-binding domain as being important in the cAMP-induced conformational change (Kim et al., 1992). We have studied further this region and found a substitution (Valine for aspartic acid at residue 138) that makes the protein allosteric defective (Ryu, S., Garges, S., and Adhya, S., manuscript submitted). The allosteric defective mutant CRP can bind cAMP normally, but does not undergo the allosteric change. We have looked for intragenic suppressors of the allosteric-defective mutant and have determined the amino acid changes in them. Second site *crp* mutants that strongly suppress the allosteric defective phenotype map at amino acids 141 and 148. We had previously identified residue 141 as potentially interaction the 138. Therefore, that the 141 Glycine to aspartic acid change could suppress a 138 defect was not unexpected. The amino acid residue at 148 faces away from the hinge and abuts the DNA-binding F α-helix. This substitution at position 148 may allow pushing of the F α-helix away the hinge area, and thus allow the normal cAMP-induced conformational change to occur. We have purified the mutant proteins and found that, indeed, the suppressor mutations allow CRP to undergo a normal cAMP-induced change.

b. The binding of CRP to DNA:

There are specific sites to which CRP can bind. These sites have dyad symmetry and there are specific bases within the site that are known by chemical and structural studies to make contact with CRP. We have previously found (Barber, A., Zhurkin, V., and Adhya, S., manuscript submitted) that an extra two base pairs in the center of the site would allow CRP to bind normally if the binding site center contained sequences that were A-philic (had a stronger tendency to exist in A-form rather than B-form). This has led us to hypothesize that since CRP binds more readily to A-philic DNA, CRP may induce a B to A transition in DNA to which it binds. A visiting scientist, Dr. Valery Ivanov, has been looking at this possibility by measuring circular dichroism. He has found that the CRP binds to its specific site, it induces a change in the DNA that could indeed, reflect a B- to A-form transition. This could have implications in the third step, that is, how CRP actually activates transcription.

c. CRP activation of transcription:

To test whether CRP activates transcription by a change in DNA that is transmitted to a nearby promoter, we have made alterations in the DNA between the *lac* CRP binding site and the *lac* promoter. We have introduced a nick, a 1-base, 2-base or 4-base gap in either strand in the region. Activation is normal when a nick or 1-base gap is present, but is decreased or absent when a 2-base or 4-base gap is present, respectively. We have shown that the defect in the 4-base gap is neither because of a failure of CRP or RNA polymerase to bind, nor because of a failure of the two proteins to exhibit cooperativity in binding. An explanation is that there is a change in DNA whose transmission to the promoter requires base stacking. This is currently being tested.

2. The role of CRP in *E. coli*:

In a collaboration with Dr. Saul Roseman at Johns Hopkins University, we have found that the *pts* operon encoding proteins necessary for sugar uptake is regulated by CRP in a novel manner. There are two CRP binding sites and both appear to be necessary for activation of the promoter. There may be interaction between the two sites (Fox et al., 1992).

We have found a class of *crp* mutants that has a phenotype not previously described. These *crp* mutants are slower-growing than a *crp* deletion and shows dominance over wild type allele. From DNA sequence analysis, all mutants in this class map early in the *crp* gene and result in polypeptide chain termination at different positions within a small segment. There is a site within *crp* that could be new translation initiation point: there is an ATP encoding methionine at residue at amino acid 59. Preceding this is a consensus Shine-Dalgarno sequence, which follow the chain termination mutations. To investigate whether the phenotype is caused by the presence of CRP protein lacking the first 58 amino acids because of translation termination at position 59, we are using CRP antibodies to determine the presence of the shorter reinitiated CRP proteins. Preliminary studies show that this protein is, indeed, present.

Publications:

Fox D, Presper K, Adhya, S, Roseman S, Garges S. Regulation of *pts* expression by two promoters and two cAMP receptor protein binding sites, Proc Natl Acad Sci USA 1992; in press.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08751-12 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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

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

PI:	S. Adhya	Chief, Developmental Genetics Section	LMB, NCI
Other:	M. Weickert	IRTA Fellow	LMB, NCI
	H. Choy	Visiting Fellow	LMB, NCI
	Y. N. Zhou	IRTA Fellow	LMB, NCI
	P. Parrack	Visiting Fellow	LMB, NCI
	S. Garges	Microbiologist	LMB, NCI
	N. Ogihara	Microbiologist	LMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Developmental Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	5.5	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.)*

Each of the two promoters of the *gal* operon of *E. coli* is negatively regulated by two repressors: the classical Gal repressor and the newly discovered Gal isorepressor. Both act by binding to the same two spatially separated operators, O_E an O_I. Gal repressor and isorepressor belong to a family of bacterial regulatory proteins, we termed GalR family. An alignment of the proteins of the family show 60% homology throughout the entire sequences. We have established that both repressor and isorepressor participate in the modulation of a *gal* regulon, which includes, besides the *gal* operon, the *mgl* operon encoding one of the galactose transport systems and *galR* and *galS*, the genes for Gal repressor and isorepressor respectively. The isorepressor has a major and the repressor a minor role on the negative control of the *mgl* operon. The latter carries a *gal* operator element at the -60 region. The effect of isorepressor on *mgl* is at the level of transcription initiation.

An *in vitro* transcription assay has been developed to study regulation with purified proteins. It uses supercoiled DNA minicircles which carry only the *gal* promoter segment. In this system, using DNA minicircle containing the *gal* promoters with *lac* operator sequences at O_E and O_I, we have demonstrated the requirement of DNA looping *in vitro*. Lac repressor, which is capable of association into a tetramer and forming a DNA loop as observed by electron microscopy, repressed transcription from *P*1 and *P*2 while a nonlooping Lac repressor mutant failed to show normal repression from both promoters. Repression mediated by DNA looping inhibited the synthesis of complete as well as abortive transcripts, demonstrating that the repression was on the formation or activity of the initial transcribing complex. DNase foot printing results indicated that repressor does not inhibit RNA polymerase binding.

Major Findings:

Negative control of *gal* regulon.

Recently we discovered that the *gal* operon of *Escherichia coli* is regulated by, besides the classical Gal repressor, an isomer, which we have termed Gal isorepressor. Both repressor and isorepressor repress *gal* operon *in vivo* by interacting with the bipartite operators, O_E and O_I. The following observations highlight our findings made during the year.

1. We have found that operator sequence that interact with repressor and isorepressor are also present in the *mgl* operon, encoding proteins for galactose transport, as well as in genes *galR* and *galS* that encode the repressor and isorepressor, respectively.

2. Transcription from different promoters of the *gal* regulon, *galP*1, *galP*2 and *mgl*P was examined by primer extension and reverse transcription of mRNA isolated from strains containing mutations in *galR* and/or *galS*. In strains containing a *galS* mutation, overexpression of *gal* message occurred only in the presence of inducer, while *mgl* message was constitutive. The *galS* mutation also derepressed the expression of an *mglA*::*lacZ* fusion, demonstrating that GalS acts as a *mgl* repressor. The *gal* and *mgl* operons constitute a regulon. Crosstalk, temporal action, induction spectrum of heteromer formation between repressor and isorepressor may help coordinate high affinity galactose transport and galactose utilization. Potential autoregulation of *galR* and *galS* genes are in progress.

3. We have constructed a phage T7 promoter based plasmid expression system in *E. coli* to overexpress the GalS protein. A very high level of a 37 Kdal protein was expressed in this system with an intact *galS* gene. The size of the protein is the same as the deduced molecular weight of the GalS protein. The overexpression will simplify purification for *in vitro* experiments.

4. We have found a family of proteins, homologous to Gal repressor, which regulate transcription of inducible genes in bacteria (GalR family). An alignment of the proteins in the GalR family show a very high degree of similarity (60%) throughout the enter sequences. The homology is greatest among the amino terminal DNA binding domain. Since a portion of the operator sequences occupied by these proteins is also conserved, a similar DNA structure may be required for specific recognition of DNA by members of the GalR family. Highly conserved motifs involved in effector biding and oligomerization are also identified. This compilation suggests a widespread conservation of these regulators among bacteria, and have strong implications for further study of peptide motifs in domain function, as well as pathways of protein evolution.

5. We have proposed that repression of the *gal* operon requires the interaction of the two operator (O_E and O_I)- bound repressors forming a DNA loop. Involvement of DNA looping between two spatially separated gal operators, O_E and O_I, in repression of the operon has been demonstrated *in vivo*. An *in vitro* transcription assay using a minicircle DNA containing the *gal* promoter with *lac* operators was employed to elucidate the molecular mechanism of repression. Lac repressors, which are capable of associating into a tetramer and forming a DNA loop, repressed transcription from *P*1 and *P*2 while a non-looping Lac repressor mutant failed to show normal repression of both of the *gal* promoters. Thus a DNA loop is also required for repression of transcription *in vitro*.

   Repression mediated by DNA looping resulted in the inhibition of the synthesis of complete as well as abortive transcripts, demonstrating that the repressive action was on the formation or

activity of the initial transcribing complex (ITC). Under similar conditions, Gal repressor did not repress the *gal* promoter effectively because it fails to loop DNA containing *gal* operators in the purified system. Component(s) or conditions that aid Gal repressor in DNA looping *in vitro* remain to be identified.

6. DNase footprinting results indicated that repressor, CRP and RNA polymerase can all be present simultaneously on the *gal* DNA. From these results we proposed that Gal repressor inhibits transcription by a direct contact with RNA polymerase. To investigate this, three *galR^s* mutants (super repressor with no *gal* expression even in the presence of inducer) were tested for derepression of the *gal* operon when each of the different subunits of RNA polymerase is overproduced. We have found that overproduction of α subunit of RNA polymerase gives a partial derepression of gal expression for one of the *galR^s* mutant, supporting the concept of repressor RNA polymerase interaction. We have sequenced all three *galR^s* mutants: 1) a Tyr to Phe change located at aa #244, a position which may interact with inducer and is partial derepressed by overproduction of α subunit of RNA polymerase. 2) a Ser to Phe change located at aa #184, near a region believed to interact with inducer. This mutant does not respond to overproduction of the γ subunit of RNA polymerase. 3) a double mutation of *galR* containing the Tyr to Phe change at aa #244 and a Val to Ala change at aa #142. This double mutant also does not respond to α overproduction.

Publications:

Golding A, Weickert MJ, Tokeson JPE, Garges S, Adhya S. A mutation defining the ultrainduction of the *Escherichia coli gal* operon, J Bacteriol 1991;173:6294-96.

Orban L, Chrambach A, Zwieb C, Adhya S. Detection of conformational and net charge differences in DNA-protein complexes by quantitative electrophoresis on polyacrylamide-Agarose copolymer gels,. Electrophoresis 1991;12:391-6.

Weickert MJ, Hogg RW, Adhya S. Location and orientation on the *Escherichia coli* physical map of the *mgl* operon and *galS*, a new locus for galactose ultrainduction, J Bacteriol 1991;173:7412-13.

Weickert MJ, Adhya S. A family of bacterial regulators homologous to Gal and Lac repressors, J Biol Chem 1992; (in press).

Weickert MJ, Adhya S. An isorepressor of the gal regulon in *Escherichia coli*, J Mol Biol 1992; (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08752-12 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Mechanisms of Thyroid Hormone Action in Animal Cells

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

PI:	Sheue-yann Cheng	Chief, Gene Regulation Section	LMB,	NCI
Others:	C. Parkison	Technician	LMB,	NCI
	K. Ashizawa	Visiting Fellow	LMB,	NCI
	J. Park	Visiting Fellow	LMB,	NCI

COOPERATING UNITS *(if any)*

Peter McPhie, Laboratory of Biochemistry and Metabolism, NIDDK
Bruce Weintraub, Molecular, Cellular and Nutritional Endocrinology Branch, NIDDK

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

To understand the molecular mechanism(s) by which the thyroid hormone, 3,3',5-triiodo-L-thyronine (T_3) promotes growth and differentiation, the structure and activity of two cellular thyroid hormone binding proteins have been studied.

I. Role of phosphorylation on the transcriptional activity of human β1 thyroid hormone nuclear receptors (h-TRβ1). h-TRβ1 was found to be a phosphoprotein *in vivo* with Ser, Thr and Tyr (85:10:5) as the phosphorylation sites. Okadaic acid, a potent inhibitor of phosphatase 1 and 2A, stimulated the phosphorylation of h-TBβ1 by 3-, 7- and 11-fold at the concentrations of 0.1, 0.25 and 0.5 μM, respectively. The increase in phosphorylation was accompanied by a concomitant increase in receptor-mediated transcription in transient transfection assays. h-TRβ1 purified from *E. coli* was phosphorylated *in vitro* by the endogenous kinase from cellular extracts. Ser, Thr and Tyr were phosphorylated in a similar ratio to that found *in vivo*. The *in vitro* phosphorylation was stimulated by okadaic acid. Phosphorylation did not affect the binding of h-TRβ1 to T_3. However, phosphorylation of h-TRβ1 resulted in an increase of its binding to DNA and conferred on it the ability to bind to nuclear accessory proteins. Taken together, these results indicate that phosphorylation plays an important role in the transcriptional activity of h-TRβ1.

II. The cytosolic thyroid hormone binding protein (p58-M_2) regulates the h-TRβ1-mediated transcription. The transcriptional activity of h-TRβ1 is T_3-dependent. To explore the possible role of p58-M_2 as a regulator for T_3 action, we evaluated the effect of the availability of cytoplasmic T_3 on the modulation of transcriptional responses of T_3 receptor. In human choriocarcinoma JEG-3 and monkey COS-1 cells, p58-M_2 is a monomer of the tetrameric pyruvate kinase, subtype M_2(PKM_2) which does not bind T_3. The *in vivo* monomer-tetramer interconversion is regulated by glucose via fructose 1,6-bisphosphate. At the physiological T_3 concentration, lowering the glucose concentration led to an increase in the cellular concentration of p58-M_2 and a concomitant reduction in the transcriptional activity of a transfected h-TRβ1 in both cell lines. In the absence of glucose, the transcriptional activity of h-TRβ1 in JEG-3 and COS-1 cells was reduced by 65-75% and 90-95%, respectively. These findings demonstrate an important prenuclear step in the modulation of the gene regulating activity of the T_3 receptors.

Major Findings:

I. Role of phosphorylation on the structure and function of thyroid hormone receptors.

A. *In vitro* phosphorylation alters DNA binding activity of h-TRβ1. One of the critical questions in understanding the mechanism of thyroid hormone action is how the binding of T_3 to domain E regulates the transcriptional activity of the domain C. Since protein phosphorylation is known as one of the principal regulatory mechanisms which controls biological processes, we addressed this issue by first examining whether h-TRβ1 can be phosphorylated by the endogenous kinase(s) *in vitro*. Furthermore, we evaluated the effects of phosphorylation on its hormone and DNA binding activity.

Using the endogenous protein kinase in the cellular extracts of HeLa cells, h-TRβ1 was found to be rapidly phosphorylated. Maximal phosphorylation was reached at ~30 min. Acid hydrolysis of the phosphorylated h-TRβ1 showed that serine, threonine and tyrosine are the phosphorylated amino acids. Studies with kinase activators or inhibitors suggest that h-TRβ1 is not phosphorylated by kinase(s) with specificity similar to cAMP-dependent protein kinase, protein kinase C and caesin kinase II. Evaluation of h-TRβ1 kinase in cell lysates indicates the following decreasing order of activity in different cell types: HeLa>GH3>JEG-3>COS-1>HepG2>NIH 3T3>Detroit 535 fibroblasts (relative ratio = 80:40:22:14:12:4:1). Binding of T_3 to h-TRβ1 was compared before and after phosphorylation. Scatchard analysis of the binding data indicates that phosphorylation has no effect either on the binding affinity or the binding capacity. However, binding of h-TRβ1 to the T_3 response element of rat growth hormone gene (r-TRE) is stimulated by two-fold. Binding to the T_3 response element was further increased by two to three-fold upon the addition of nuclear accessary proteins. The effect of phosphorylation is reversible. Treatment of the phosphorylated h-TRβ1, with either potato acid phosphatase or alkaline phosphatase dephosphorylated h-TRβ1. Removal of Pi resulted in the loss of r-TRE binding activity. These results indicate that phosphorylation confers the ability of h-TRβ1 to bind to the nuclear accessory proteins.

B. *In vivo* phosphorylation stimulates the transcriptional activity of h-TRβ1. COS-1 cells which were transfected with the expression vector of h-TRβ1 were pulsed with [^{32}P]orth-phosphoric acid and immunoprecipitated with monoclonal antibody J52. *In vivo* h-TRβ1 was found to be a phosphoprotein. In the presence of 0.1, 0.25 and 0.5 μM of okadaic and phosphorylation of h-TRβ1 was dramatically increased by 3-, 7-, and 11-fold, respectively. The increase in phosphorylation was not due to the increase in the rate of synthesis of h-TRβ1. The effect of phosphorylation on the regulation of h-TRβ1-mediated transcription was assessed by co-transfection of h-TRβ1 expression plasmid and a reporter gene that contained the multiple copies of T_3 response element in tandem upstream of the chloramphenicol acetyltransferase (CAT) gene. Concomitant with the increase in the phosphorylation of h-TRβ1, the h-TRβ1-mediated transcription was also increased in a concentration-dependent manner. At 0.5 μM okadaic acid, a 2.2-fold increase in the receptor-mediated transcription was detected. This increase, however, was not a result of a general increase in the transcriptional activity of cells. Taken together, these results indicate that the transcriptional activity of h-TRβ1 is regulated by phosphorylation.

II. Functional regulation of cytosolic thyroid hormone binding protein (CTHBP).

A. p58-M_2 regulates the T_3 transcriptional activity. The findings that *in vivo* formation of the monomeric p58-M_2 is glucose-dependent provided us with a means to evaluate the physiological role of p58-M_2. We compared the transcriptional activity of transfected h-TRβ1

by T_3 in the absence or presence of glucose in monkey COS-1 and human choriocarcinoma JEG-3 cells. In monkey COS-1 and JEG-3 cells, monomeric p58-M_2 and the tetrameric PKM_2 interconversion was found to be regulated by glucose via Fru-1,6-P_2. The transcriptional activity of h-TRβ1 was measured using the reported chloramphenicol acetyltransferase (CAT) gene whose expression is controlled by T_3 response element. The CAT activity decreases as the glucose concentration drops, indicating that as the formation of p58-M_2 is increased, more T_3 is bound to p58-M_2. Therefore, less T_3 is available to h-TRβ1 to mediate the T_3-dependent transcriptional activity.

The reduction in the transcriptional activity of h-TRβ1 resulting from glucose deprivation was not due to a general decrease in basal transcription. Glucose has no effect on the CAT activity whose expression is under the control of a SV40 promoter, a herpes simplex virus thymidine kinase, or a mouse mammary tumor virus promoter. We have also examined glucocorticoid receptor-mediated transcription under identical conditions and no significant effect of glucose was found. These results indicate that p58-M_2 regulates the transcriptional activity of T_3 by acting as a cytosolic buffer for T_3. Thus, p58-M_2 acts as a prenuclear regulator in the modulation of the gene regulatory activity of the T_3 receptor. As far as we know, our study is the first report to demonstrate the regulatory function of a cytosolic T_3 binding protein.

B. p58-M_1 is regulated by T_3 at the transcriptional level. We have recently shown that the monomer of pyruvate kinase subtype M_1 is also a thyroid hormone binding protein. To understand the role p58-M_1 plays in thyroid hormone action, we examined the regulation of p58-M_1 by T_3 in GH_3 cells. Expression of p58-M_1 was evaluated by metabolically labeling GH_3 cells cultured in regular medium, thyroid hormone-depleted medium (T_d - medium) or T_d medium supplemented with T_3 (T_d + T_3 medium) followed by immunoprecipitation. T_3 stimulates the expression of p58-M_1 by two-fold. Analysis by pulse-chase experiments indicates that the increased expression is not due to the increased stability of p58-M_1. Northern analysis of mRNA prepared from cells cultured in regular T_d or T_d + T3 medium demonstrates that T_3 increases the accumulation of cytoplasmic mRNA by two-fold. Nuclei from cells cultured in these conditions were prepared and the rates of synthesis of nascent nuclear RNA were compared by an *in vitro* transcription assay. Addition of T_3 stimulates the rate of transcription by two-fold. The parallel and identical magnitude in the increase of transcription rate and the accumulation of mRNA indicate the T_3 stimulates the synthesis of p58-M_1 by increasing the transcriptional activity of its gene.

Publications:

Lin K-h, Cheng S-y. An efficient method to purify active eukaryotic proteins from the inclusion bodies in *Escherichia coli*, Biotechniques 1991, 6: 748-750.

Lin K-h, Cheng S-y. Zn(II) and other divalent cations induce reversible cross-linking of human α1 nuclear receptor with no effect on hormone binding, Proc 10th International Thyroid Congress 1991;865-68.

Ashizawa K, Willingham MC, Liang C-M, Cheng S-y. *In vivo* regulation of monomer-tetramer conversion of pyruvate kinase, subtype M_2, by glucose is mediated via fructose-1,6-bisphosphate, J Biol Chem 1991;264:16842-46.

Parkison C, Ashizawa K, McPhie P, Lin K-h, Cheng S-y. The monomer of pyruvate kinase, subtype M_1, is both a kinase and a cytosolic thyroid hormone binding protein, Biochem. Biophys Res Commun 1991;179:668-674.

Meier C A, Dickstein BM, Ashizawa K, McClaskey JH, Muchmore P, Ransom SC, Merk JB, Hao EU, Usala SJ, Bercu BB, Cheng S-y, Weintraub BD. Variable transcriptional activity and ligand binding of mutant β1 3,3',5-triiodo-L-thyronine receptors from four families with generalized resistance to thyroid hormone, Mol Endo 1992;6:248-58.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08753-10 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Immunotoxin and Recombinant Toxin Therapy of Cancer

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

PI: I. Pastan Chief, Laboratory of Molecular Biology NCI

Co-investigator: D. FitzGerald Microbiologist LMB, NCI

COOPERATING UNITS *(if any)*

BioCarb, Gaithersburg, MD; Cardiology Br., NHLBI, NIH; American Red Cross, Rockville, MD; Fox Chase Cancer Center, Philadelphia, PA; BRMP, FRCF, Frederick, MD; Duke U. Medical Center; ; U. CA, San Diego, La Jolla, CA; Lab. Immunol., NEI, NIH; Lab. Develop. & Mol. Immunity, NICHHD, NIH; Div. Hematol.-Oncol., U. Arkansas Sciences, Little Rock, AZ

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
18.0	14.0	4.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Pseudomonas exotoxin and genetically modified forms of PE have been attached to monoclonal antibodies (mAbs) or growth factors to create cell specific cytotoxic agents. A mutant form of PE, LysPE38, has been chemically attached to mAb B3. This immunotoxin, B3-LysPE38, causes regression of human tumors in mice with a large therapeutic window. Clinical grade mAb B3 has been prepared and clinical grade LysP38 is being prepared for an immunotoxin trial scheduled to begin in 1992. Other mutant forms of LysPE38 have been evaluated for the production of immunotoxins, and one in which the lysine residues at the end domain III have been mutated makes a more active immunotoxin. A single chain immunotoxin (B3(Fv)-P38 KDEL) has been prepared and also causes tumor regression in mice. The conditions of producing B3(Fv)-P38 KDEL have been improved by studying refolding conditions and using mutant linker and connector molecules. Refolding can also be stimulated by the enzymes GroE and protein disulfide isomerase. The side chains of the amino acids in domain II have been changed to alanine residues to determine which side chains are important for the cytotoxic action of PE. Single domain immunotoxins have also been made with B3(Fv)-P38 KDEL and the results show that both the light and heavy chain interact with the B3 antigen. Indium labeled B3 has been prepared and shown to image tumors very well in nude mice. Single chain recombinant immunotoxins have been made with antibodies that react with the erbB2 oncogene, with an antibody C242 that binds with many human colon cancers and with antiTac that binds to the IL2 receptor present in many lymphomas and leukemias. These agents are in preclinical development. Other immunotoxins have been prepared that react with B cell lymphomas and the p75 subunit of the IL2 receptor. Recombinant toxins with longer half lifes have been created by inserting the C_H2 domain of human IgG1 between the ligand and the toxin domains.

Other Personnel:

W. Debinski	Special Volunteer	LMB,	NCI
L. Pai	Medical Staff Fellow	LMB,	NCI
T. Prior	Visiting Fellow	LMB,	NCI
R. Kreitman	Clinical Associate	LMB,	NCI
U. Brinkman	Visiting Fellow	LMB,	NCI
K. Kasturi	Visiting Fellow	LMB,	NCI
Q.-C. Wang	Visiting Associate	LMB,	NCI
C. Theuer	Research Associate	LMB,	NCI
A. Kihara	Visiting Fellow	LMB,	NCI
E. Mesri	Special Volunteer	LMB,	NCI
K. Webber	Senior Staff Fellow	LMB,	NCI
M. Choe	Special Volunteer	LMB,	NCI
J. Buchner	Visiting Fellow	LMB,	NCI
S. Kunwar	Howard Hughes Fellow	LMB,	NCI
E. Lovelace	Biologist	LMB,	NCI
M. Gallo	Microbiologist	LMB,	NCI
B. Yu	Special Volunteer	LMB,	NCI
A. Harris	Biological Laboratory Technician	LMB,	NCI

Major Findings:

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

Immunotoxins are prepared by attaching mutant forms of *Pseudomonas* exotoxin (PE) to antibody molecules that target cancer cells. Two types of immunotoxins have been made. The first type is a conventional immunotoxin in which an antibody or an Fab' fragment of an antibody is attached by a chemical linkage to a recombinant form of PE. The principle recombinant form of PE is one in which domain I, the cell binding domain of PE, is removed to produce a protein with a molecular weight of 40,000 termed PE40. PE40 has been engineered in various ways in order to make it more useful when attached to PE. One of these variants, LysPE40, has an extra lysine residue placed at its amino terminus which is used to couple PE40 to the antibody. A second variant has a deletion of amino acids 365-380 producing a smaller mutant form termed LysPE38 because its molecular weight is about 38,000. Other mutant forms have the carboxyl end of the molecule modified. One of these modifications converts the carboxyl end of PE from REDLK to KDEL which makes a more active toxin. A second modification is the elimination of lysine residues at the end of domain III. This molecule termed LysPE38QQR has two lysine residues converted to glutamine and the terminal lysine residue converted to arginine.

Two monoclonal antibodies which react preferentially with human cancer cells are under development. The first of these, mAb B3, reacts with many human cancers including cancers of the colon, breast, ovary, lung, stomach, esophagus and bladder. This antibody also reacts with several normal tissues including glands of the stomach and the epithelium of the trachea and bladder. Because of the wide reactivity of mAb B3 with human cancers, L. Pai and U. Brinkmann have developed an immunotoxin with this agent and shown that B3-LysPE40 kills many types of cancer cells and causes complete regression of antigen bearing tumors in mice. To select a drug for a clinical study, we attached several recombinant forms of PE40 and PE38 to mAb B3. All of

these immunotoxins caused complete regression of tumors in mice but the therapeutic window varied. The IT with the best therapeutic window is B3-LysPE38. Because monkeys have the same distribution of the B3 antigen as humans, we have performed preclinical toxicity studies in monkeys with B3-LysPE38 and have found that monkeys can be given 1 mg/kg intravenously every other day for three doses without liver toxicity or toxic effects on stomach, bladder or trachea. This dose is therapeutic in mice and we are encouraged by these studies. We will soon meet with the FDA to obtain approval for a clinical study. Clinical grade antibody has been made for this study and we are in the process of making the clinical grade recombinant LysPE38.

B3-LysPE38 is a large molecule with a molecular weight of 200,000. Its composition is heterogeneous because the positions at which the toxin and antibody are chemically attached cannot be controlled. In addition, the large size of the IT may inhibit tumor penetration. To overcome these difficulties and make a molecule which can be produced more economically, U. Brinkmann has made a recombinant toxin in *E. coli* in which the variable region of mAb B3 in the single chain form is attached to PE38. This molecule termed B3(Fv)-P38 can be produced in *E. coli* and when administered to mice intravenously will cause complete regression of a human epidermoid carcinoma. One problem with this molecule is that it is difficult to produce in large amounts because the recombinant toxin aggregates during purification.

We have been studying the basis of this aggregation and believe it is due to some interaction of the Fv fragment with PE38. U. Brinkmann and R. Kreitman have made several mutant forms of the toxin to try and diminish this interaction. We have prepared different "linker" peptides between the heavy and light chain and also prepared different "connector" peptides that connect the light chain to the toxin. One of these connectors, termed C3, results in a 2-3 fold increase in yield due to diminished aggregation. We have also analyzed the conditions required for optimal refolding of recombinant toxins and developed a protocol using a redox reshuffling buffer which increases the yield of properly folded molecules. In addition, molecular chaperones and protein disulfide isomerase stimulate proper refolding.

A second problem with PE based immunotoxins is non-specific liver toxicity which we believe results from a low affinity interaction of some portion of PE40 with the surface of hepatocytes. To address this question, S. Kasturi has begun to mutate or remove surface residues in domain II, Ib and III. In one series of studies, many of the surface residues in domain II were changed to alanine. The results of these studies indicate that many residues on the surface of domain II are important for the cytotoxic action of PE and cannot be changed to alanine without moderate to major loss of activity. However, several mutant forms retain full activity and these will now be assessed for non-specific liver toxicity. In addition, the inactive alanine mutants will have their side chains altered in another manner to examine the effect of these changes on the cytotoxic action of PE and the non-specific cytotoxic activity of PE40. A second approach to diminish non-specific cytotoxicity and which also should reduce immunogenicity is to derivatize amino groups on the toxin with polyethylene glycol. Q. Wang has initiated these studies with a variant of TGFα-PE40 in which the lysine residue on TGFα has been changed to arginine to prevent its derivatization and a spacer placed between TGFα and PE40. Preliminary studies indicate that a mutant form of TGFα-PE40 that contains about 11 lysine residues can be derivatized with polyethylene glycol to produce a derivatized molecule with a prolonged half life in the blood. The effect of this change on non-specific binding to liver is currently under study.

U. Brinkmann has prepared single domain recombinant toxins with B3 in which either the heavy chain variable region or the light chain variable region is fused to PE38. Both of these molecules kill target cells although they are about 50-fold less active than the Fv-toxin. These results indicate both the light chain and heavy chain of B3 bind to antigen.

In collaboration with O. Gansow and J. Carrasquillo, L. Pai has made an indium-labeled chelate of B3 which images tumors in nude mice and has low uptake by the liver. We have also made a recombinant Fab form of B3 in *E. coli* and plan to use it to perform further imaging studies. Several other antibodies are being developed as possible immunotoxins. One of these is mAb C42, in collaboration with Kabi Pharmacia. C242 reacts with more than 70% of human colorectal cancers. C242-LysPE40 was prepared by W. Debinski and was found to be active *in vivo* causing significant retardation of the colon cancer xenograft, COLO 205. A recombinant single chain immunotoxin has recently been prepared with C242 and is currently under evaluation.

J. Batra and A. Kihara have studied antibodies to the erbB2 oncogene in collaboration with Molecular Oncology. Four antibodies were evaluated as immunotoxins and one of these, e23, chosen for further study. e23(Fv)-PE38 KDEL is extremely cytotoxic to target cells expressing the erbB2 antigen and causes regression of tumors with this antigen when administered to nude mice. Several different forms of e23(Fv)-PE have been prepared in order to choose a molecule for a preclinical trial. R. Kreitman has prepared Mikβ1(Fv)-PE40 directed at the p75 subunit of the IL2 receptor. This protein is cytotoxic to YT-S cells which only express this subunit of the IL2 receptor.

LL2 is an antibody supplied by ImmunoMedics which reacts with B cell lymphomas and leukemias. R. Kreitman has prepared chemical conjugates of LL2 and PE and shown these kill target cells in culture and cause tumor regression in animals. To improve the efficacy of chemical conjugates, recombinant forms of PE38 have been prepared in which the lysine residues at the end of domain III have been changed to glutamine or deleted. Chemical conjugates prepared with these molecules have a somewhat higher activity and appear to have a larger therapeutic window in animals. In addition, W. Debinski has studied the activity of immunotoxins made with Fab' fragments of antibodies utilizing the SH group in the Fab' for coupling. He has found that one can obtain very active immunotoxins which may be useful because their small size allows them to penetrate tumors better.

Single chain immunotoxins and growth factor toxin fusions have a short half life. To prepare molecules with longer half lifes. J. Batra and S. Kasturi have inserted the C_H2 domain of human IgG1 between the ligand binding domain and PE40 to make CD4-C_H2-PE40 and TGFα-C_H2-PE40. In addition, other portions of the constant region have been inserted into CD4-PE40. CD4-C_H2-PE40 has the longest half life in animals and retains full activity on target cells.

Various types of indirect evidence have suggested that domain II of PE is sufficient to translocate domain III into target cells. To obtain direct evidence about this question, T. Prior has replaced domain Ib and domain III of PE (aa 365-600) with barnase, a small bacterial ribonuclease. He has found that PE barnase is a very cytotoxic molecule as long as the carboxyl terminus, REDLK, is retained. Therefore, we are confident that amino acids 253-364 are sufficient to translocate domain III or molecules inserted in its place into target cells.

C. Theuer has been investigating how domain II functions. Using information generated by Ogata and FitzGerald that a cleavage between arginine 279 and glycine 280 generates a 37 kilodalton fragment that is translocated to the cytosol, he has prepared the 37 kilodalton fragment (290-613) in *E. coli* and directed it to target cells by insertion of TGFα just proximal to its carboxyl terminus. This construct termed PE37/TGFα is very cytotoxic to EGF receptor bearing cells. In addition, C. Theuer has prepared the PE(290-613) with a free SH group at position 287 and used this SH group to make a disulfide bond with various antibodies. This form of PE is very similar to ricin A chain in which the free SH group is used to couple it to antibodies.

R. Kreitman has evaluated the activities of antiTac(Fv)-PE40 and various derivatives on cells from patients with adult T cell leukemia and lymphoma and shown that this molecule is cytotoxic to cancer cells directly isolated from patients. R. Kreitman has also investigated the activity of an IL6-PE molecule against bone myeloma cells in the marrow of patients with multiple myeloma and shown that many of these cells are targets for this chimeric toxin.

E. Mesri and R. Kreitman have prepared a chimeric toxin in which the heparin binding domain of heparin binding EGF has been ligated to transforming growth factor α. This new chimeric toxin binds strongly to heparin and to the EGF receptor and is cytotoxic to cells containing EGF receptors. The activity of this new toxin on other cell lines is currently under study.

Publications:

Chaudhary VK, Moss B, Berger EA, FitzGerald DJ, Pastan I. CD4-PE40 - A chimeric toxin active against human immunodeficiency virus (HIV)-infected cells. In Gallo RC, Gilbert J, eds. The Human Retroviruses. Orlando: Academic Press, 1991;379-87.

Siegall CB, Ogata M, Pastan I, FitzGerald DJ. Analysis of sequences in domain II of *Pseudomonas* exotoxin A which mediate translocation, Biochem 1991;30:7154-9.

Pastan I, Lovelace ET, Gallo MG, Rutherford AV, Magnani JL, Willingham MC. Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. Cancer Res 1991;51:3781-7.

Pastan I, Willingham MC, Gottesman MM. Molecular manipulations of the multidrug transporter: A new role for transgenic mice, FASEB J 1991;5:2523-7.

Epstein SE, Siegall CB, Biro S, Fu Y-M, FitzGerald D, Pastan I. Cytotoxic effects of a recombinant chimeric toxin on rapidly proliferating vascular smooth muscle cells, Circulation Res 1991;84:778-87.

FitzGerald D, Pastan I. Redirecting *Pseudomonas* exotoxin. In Lord JM ed.Seminars in Cell Biology. Philadelphia: Saunders Scientific Publications, 1991;31-7.

Seetharam S, Chaudhary V, FitzGerald D, Pastan I. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL, J Biol Chem 1991;266:17376-815.

Siegall CB, Epstein S, Speir E, Hla T, Forough R, Maciag T, FitzGerald D, Pastan I. Cytotoxic activity of chimeric proteins composed of acidic fibroblast growth factor and *Pseudomonas* exotoxin on a variety of cell types, FASEB J 1991;5:2843-9.

Brinkmann U, Pai LH, FitzGerald DJ, Pastan I. B3(Fv)-PE38KDEL, a single chain immunotoxin that causes complete regression of a human carcinoma in mice, Proc Natl Acad Sci USA 1991;88:8616-20.

Pai LH, Bookman MA, Ozols RJ, Young RC, Smith JW II, Longo DL, Gould B, Frankel A, McClay EF, Howell S, Reed E, Willingham MC, FitzGerald DJ, Pastan I. Clinical evaluation of intraperitoneal *pseudomonas* exotoxin immunoconjugate OVB3-PE in patients with ovarian cancer, J Clin Oncol 1991;9:2095-103.

Willingham MC, Pastan I. Endocytosis - An Overview. In: Steer CJ, Hanover J, eds. Intracellular Trafficking of Proteins. England: Cambridge University Press, 1991:3-14.

FitzGerald D, Pastan I. Pseudomonas exotoxin and derived conjugates: Interactions with mammalian cells. In: Steer CJ, Hanover J, eds. Intracellular Trafficking of Proteins. England: Cambridge University Press, 1991:226-46.

Pastan I. Recombinant toxins directed toward the IL-2 receptor. In: Waldmann TA, Moderator. The multichain interleukin-2 receptor: A target for immunotherapy. Annals of Internal Medicine. 1991:148-60.

Pastan I, FitzGerald D. Oncotoxins for cancer treatment, Science 1991;254:1173-7.

Herbort CP, de Smet MD, Roberge FG, Nussenblatt RB, FitzGerald D, Lorberboum-Galski H, Pastan I. Treatment of corneal allograft rejection with the cytotoxin IL2-PE40, Transplantation 1991;52:470-4.

Chaudhary VK, FitzGerald DJ, Pastan I. A proper amino terminus of Diphtheria toxin is important for cytotoxicity, Biochem Biophys Res Commun 1991;180:545-51.

Beraud E, Lorberboum-Galski H, Chan CC, FitzGerald D, Pastan I, Nussenblatt RB. Immunospecific suppression of encephalitogenic activated T lymphocytes by chimeric cytotoxin IL-2-PE40, Cell Immunol 1991;133:379-89.

Kreitman RJ, Siegall C, Chaudhary VK, FitzGerald DJ, Pastan I. Properties of chimeric toxins with two recognition domains: Interleukin 6 and transforming growth factor alpha at different locations in *Pseudomonas* exotoxin, Bioconjugate Chem 1992;3:63-8.

Kreitman RJ, Chaudhary VK, Siegall CB, FitzGerald DJ, Pastan I. Rational chemotherapeutic design of *Pseudomonas* exotoxin: An intramolecular location for the insertion of transforming growth factor alpha as a targeting ligand, Bioconjugate Chem 1992;3:58-622.

Fattom A, Shiloach J, Bryla D, FitzGerald D, Pastan I, Karakawa WW, Robbins JB, Schneerson R. Comparative immunogenicty of conjugates composed of the *Staphylococcus aureus* type 8 capsular polysaccharide bound to carrier proteins by adipic acid dihydrazide of N-succinimidyl-3-(2-pyridyldithio)-propionate, Infect Immun 1992;60:584-9.

Kreitman RJ, Siegall CB, FitzGerald DJP, Epstein J, Barlogie B, Pastan I. Interleukin 6 fused to a mutant form of *Pseudomonas* exotoxin kills malignant cells from patients with multiple myeloma, Blood 1992;79:177580.

Prior TI, FitzGerald DJ, Pastan I. Translocation mediated by domain II of *Pseudomonas* exotoxin A: Transport of barnase into the cytosol, Biochem 1992;31:3555-9.

Brinkmann U, Buchner J, Pastan I. Independent domain folding of *Pseudomonas* exotoxin and single-chain immunotoxins: Influence of interdomain connections, Proc Natl Acad Sci USA 1992;89:3075-9.

Brinkmann U, Pai L, FitzGerald D, PastanI. Alteration of a protease sensitive region of *Pseudomonas* exotoxin prolongs its survival in the circulation of mice, Proc Natl Acad Sci USA 1992;89:3065-9.

Pastan I, Chaudhary V, FitzGerald DJ. Recombinant toxins as novel therapeutic agents, Annu Rev Biochem (in press, 1992).

Kreitman RJ, FitzGerald D, Pastan I. Targeting growth factor receptors with fusion toxins, Int J Immunopharmacol (in press, 1992).

Buchner J, Brinkmann U, Pastan I. Renaturation of a single-chain immunotoxin facilitated by chaperones and protein disulfide isomerase, Bio/Technology (in press, 1992).

Buchner J, Pastan I, Brinkmann U. A method to increase the yield of properly folded recombinant fusion proteins, *e.g.*, single-chain immunotoxins, from renaturations of bacterial inclusion bodies, Analytical Biochem, (in press, 1992).

Batra JK, Kasprzyk PH, Bird RE, Pastan I, King CR. Recombinant anti-erbB-2 immunotoxins containing *Pseudomonas* exotoxin, Proc Natl Acad Sci USA (in press, 1992).

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08754-09 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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:	I, Pastan	Chief, Laboratory of Molecular Biology	LCB, NCI
Co-PI:	M. M. Gottesman	Chief, Laboratory of Cell Biology	LMB, NCI

COOPERATING UNITS *(if any)*

A. Nienhuis, National Heart, Lung, Blood Institute, NIH

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12.0	12.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Resistance to chemotherapy of human cancers has been studied by establishing *in vitro* and *in vivo* genetic systems which mimic development of drug resistance. A major mechanism of multidrug resistance in human cancer is expression of the *MDR*1 gene which we have cloned and sequenced. Expression of the *MDR*1 gene carried by retroviral vectors can be used to transduce cells to multidrug resistance and has been used to confer resistance to mouse bone marrow cells *in vitro* and *in vivo*. Mechanistic studies on P-glycoprotein suggest that it is involved in removing drug directly from the plasma membrane. To study mechanism, we have generated many mutants and initiated biochemical studies including the purification of P-glycoprotein and reconstruction of drug-dependent ATPase activity in artificial liposomes.

Other Professional Personnel:

S. Altuvia	Special Volunteer	LCB, NCI
S. Goldenberg	Research Biologist	LCB, NCI
U. Germann	Visiting Fellow	LCB, NCI
I. Lelong	Visiting Fellow	LCB, NCI
P. Schoenlein	Staff Fellow	LCB, NCI
D.-W. Shen	Visiting Associate	LCB, NCI
I. Aksentijevich	Staff Fellow	LCB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
J. Campain	Special Volunteer	LMB, NCI
G. Evans	Biotechnology Fellow	LMB, NCI
W. Stein	Special Volunteer	LMB, NCI
M. Siegsmund	Special Volunteer	LMB, NCI
J. Aram	Special Volunteer	LMB, NCI

Major Findings:

1. Transgenic mice which express the human *MDR*1 gene in their bone marrow have been used to evaluate various techniques for sensitizing multidrug resistant cells to chemotherapy. The assay system involves intraperitoneal injection of various agents, followed by a chemotherapeutic drug (usually daunorubicin, doxorubicin, or taxol) and a determination of peripheral white blood counts (WBC) 3 and 5 days after treatment. Resistant animals show no drop in WBC under these conditions, whereas animals sensitized to chemotherapy show drops in WBC. Under these assay conditions, verapamil is a potent chemosensitizing agent, and combinations of drugs such as quinine or quinidine with verapamil appear to be synergistic. Encapsulation of doxorubicin in liposomes improves the efficacy of this drug against MDR bone marrow. The monoclonal antibody MRK-16 can reverse multidrug resistance in vivo, but this effect is not synergistic with reversing effects of lower molecular weight agents such as verapamil. The immunotoxin MRK-16-PE (*Pseudomonas* enterotoxin) effectively kills MDR bone marrow in this model.

2. MDR bone marrow from transgenic mice can be transplanted into drug sensitive mice with complete transfer of the drug resistance phenotype. This result demonstrates that resistance resides in the bone marrow cells themselves, and suggests that the *MDR*1 gene may be an effective dominant selectable marker in gene therapy experiments. To test this hypothesis, isolated mouse bone marrow cells were infected with an *MDR*1 retrovirus and reintroduced into W/W-V mice which have hypoplastic bone marrow. Presence of the *MDR*1 gene in reconstituted animals could be detected for several months. Selection *in vivo* with taxol resulted in an increase in the number of peripheral neutrophils carrying the human *MDR*1 gene, indicating that these cells had a selective advantage in the presence of the cytotoxic drug taxol.

3. KB cell lines selected for resistance to the MDR drugs colchicine or vinblastine have amplified copies of the *MDR*1 gene. Pulsed field gel electrophoretic analysis of these MDR cells at various levels of selections demonstrates two different mechanisms of amplification, both of which proceed via extrachromosomal elements to form double minute chromosomes. In the first mechanism, found in colchicine selected cells, the earliest steps of amplification appear to involve small, cytogenetically non-detectable extrachromosomal elements of approximately 890 kb. As selection (and amplification) proceeds, this element doubles in size to approximately 1800 kb (which is cytogenetically detectable as a minute chromosome), and then doubles again to 3600 kb. Restriction digestion with Not 1, which cleaves only once in the *MDR*1 amplicon, proves that this size increase results from two sequential dimerizations. In the second mechanism, seen in vinblastine-selected cells, the initial event is a formation of high molecular weight

extrachromosomal element, which decreases in size and increases in copy number during selection in vinblastine.

4. P-glycoprotein has been purified to near homogeneity from plasma membrane vesicles prepared from MDR cell lines. The purification procedure involves solubilization in octyl glucoside, followed by column chromatography with DEAE and WGA. During this procedure, extraneous ATPase activities, including the Na/K ATPase found in plasma membrane, are separated from P-glycoprotein. The purified P-glycoprotein has some basal ATPase activity (approximately 1 μmole/min/mg protein). When reconstituted into liposomes prepared from *E. coli* lipids, phosphatidyl serine, phosphatidyl choline and cholesterol, this basal activity can be stimulated several-fold by a variety of drugs known to be substrates for the P-glycoprotein efflux pump, including vinblastine and verapamil. Vanadate blocks both basal and stimulated ATPase activity.

5. Vesicles from MDR cells transport vinblastine in an ATP dependent manner. Addition of radioactive ATP or GTP results in phosphorylation of P-glycoprotein, predominantly on Ser residues. Non-radioactive GTP stimulates phosphorylation by ATP by an unknown mechanism. This phosphorylation of P-glycoprotein appears to be due to an exogenous kinase because purified P-glycoprotein does not autophosphorylate itself to any significant extent. Both protein kinase C and protein kinase A inhibitors have no effect on the exogenous phosphorylation of P-glycoprotein in this system, but staurosporine is a potent inhibitor, and vanadate stimulates phosphorylation. These results suggest the possibility that there is a novel kinase in plasma membrane vesicle preparations which phosphorylates P-glycoprotein.

6. We have continued a molecular genetic analysis of P-glycoprotein to define functional parts of the protein. Chimeras in which the first intracytoplasmic loop of P-glycoprotein is replaced by the homologous region from the *MDR*2 gene are non-functional. Replacement of 4 of 13 altered amino acids in these chimeras restores function. This result indicates that *MDR*2 does not differ that significantly from *MDR*1, and that it should be possible to use chimeras to define important functional regions of these two proteins. Substitution of the *MDR*2 amino terminal ATP site into *MDR*1 produces a fully functional transporter, indicating that this site in *MDR*2 is active in energy transduction.

7. We have inactivated one of two *mdr*1b genes in mouse adrenal Y-1 cells by homologous recombination. The *mdr*1b gene is normally expressed at high levels in these steroid-secreting cells, and we have speculated that it might be involved either in steroid secretion, or in protecting Y-1 membranes from the toxic effects of high concentrations of steroids. One *mdr*1b gene knock-out was obtained in which the second *mdr*1b allele was expressed at elevated levels compared to Y-1 parental cells. These results appear to provide a provocative association between *mdr*1b mRNA levels and steroid secretion in Y-1 cells.

8. VP-16 (etoposide) resistant human FEM-X melanoma cells have been isolated in several steps. They are cross-resistant to topoisomerase II active drugs, but not to other MDR drugs. However, isolated topoisomerase II has normal activity and is normally sensitive to VP-16 and other inhibitors. Resistance of topoisomerase II can be demonstrated in whole cells or in whole cell homogenates, but not in isolated nuclei. These results suggest that VP-16 resistance in these melanoma cells may be due to altered cellular bioavailability of VP-16, as could occur from metabolism, altered nuclear uptake, or compartmentalization.

9. High level cis-platinum resistant human KB cells and human hepatoma cells have been isolated. Cis-platinum resistance is associated with altered expression of many proteins as detected on 2D gels. Resistance from the human hepatoma cells can be transferred by DNA-

mediated gene transfer into mouse Balb/c 3T3 cells where it appears to be linked to transfer of human repetitive Alu sequences, suggesting that this linkage might be used to isolate the human genes critical for development of cis-platinum resistance.

Publications:

Cenciarelli C, Currier SJ, Willingham MC, Thiebaut F, Germann UA, Rutherford AV, Gottesman MM, Barca S, Tombési M, Morrone S, Santoni A, Marianti M, Ramoni C, Dupuis ML, Cianfriglia M. Characterization by somatic cell genetics of a monoclonal antibody to the *MDR*1 gene product (P-glycoprotein): determination of P-glycoprotein expression in multi-drug resistant KB and CEM cell variants. Int J Cancer 1991;47:533-43.

Pastan I, Willingham MC, Gottesman, MM. Molecular manipulations of the multidrug transporter: a new role for transgenic mice. FASEB J 1991;5:2523-8.

Mickisch GH, Pastan I, Gottesman MM. Multidrug resistant transgenic mice as a novel pharmacologic tool. BioEssays 1991;13:381-7.

Gottesman MM, Pastan I. The multidrug resistance (*MDR*1) gene as a selectable marker in gene therapy. In Cohen-Haguenauer O, Boiron M, eds. Human Gene Transfer Vol 219. Colloque: INSERM/John Libbey Eurotext Ltd, 1991;185-91.

Pearson JW, Fogler WE, Volker K, Usui N, Goldenberg SK, Gruys E, Riggs CW, Komschlies K, Wiltrout RH, Tsuruo T, Pastan I, Gottesman MM, Longo DL. *In vivo* administration of MRK-16 monoclonal antibody reverses drug resistance in a human colon cancer xenograft expressing the *MDR*1 cDNA. J Natl Cancer Inst 1991;83:1386-391.

Mickisch GH, Licht T, Merlino GT, Gottesman MM, Pastan I. Chemotherapy and chemosensitization of transgenic mice which express the human multidrug resistance gene in bone marrow: efficacy, potency and toxicity. Cancer Res 1991;51:5417-24.

Gottesman MM, Schoenlein P, Currier S, Bruggemann E, Pastan I. Biochemical basis for multidrug resistance in cancer. In: Pretlow G II and Pretlow P, eds. Biochemical and Molecular Aspects of Selected Cancers. San Diego: Academic Press Inc, 1991;339-71.

Lelong IH, Guzikowski AP, Haughland RP, Pastan I, Gottesman MM, Willingham MC. A fluorescent verapamil derivative for monitoring activity of the multidrug transporter. Mol Pharmacol 1991;40:490-4.

Mickisch GH, Aksentijevich I, Schoenlein PV, Goldstein LJ, Galski H, Stahle C, Sachs DH, Pastan I, Gottesman MM. Transplantation of bone marrow cells from transgenic mice expressing the human *MDR*1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice. Blood 1992;79:1087-1093.

Gottesman MM, Horio M, Lelong I, Handler J, Raviv Y, Galski H, Mickisch G, Merlino G, Willingham MC, Pastan I. Function of the multidrug transporter. In: Tsuruo T, Ogawa M, eds. Drug Resistance as a Biochemical Target in Cancer Chemotherapy. Bristol-Myers Squibb Symposia Series, San Diego: Academic Press Inc, 1991;45-62.

Gottesman MM, Pastan I. The multidrug transporter as a novel target in cancer chemotherapy. The Origins of Human Cancer: A Comprehensive Review. Cold Spring Harbor Symposium, September 4-10, 1990, New York: Cold Spring Harbor Laboratory, 1991;845-53.

Mickisch GH, Merlino GT, Alken PM, Gottesman MM, Pastan I. New potent verapamil derivatives that reverse multidrug resistance in human renal carcinoma cells and in transgenic mice expressing the human *MDR*1 gene. J. Urol. 1991;146:447-53.

Gottesman MM, Roninson IB, Pastan I. A molecular basis for multidrug-resistance in human cancer cells. In Kessel D, ed. Resistance to Antineoplastic Drugs. CRC Press: Boca Raton, FL 1989;19-35.

Goldstein LJ, Gottesman MM, Pastan I. Expression of the *MDR*1 gene in human cancers. Ozols RF, ed. Drug resistance, cancer treatment and research. Boston: Kluwer Academic Publishers 1991;101-19.

Gottesman MM, Pastan I. The multidrug transporter as a novel target in cancer chemotherapy. In: Brugge, J., Curran ,T., Harlow E, McCormick F, eds. The Origins of Human Cancer: A Comprehensive Review. Cold Spring Harbor Symposium, September 4-10, 1990. New York, Cold Spring Harbor Laboratory, 1991;845-;53.

Chin K-V, Ueda K, Pastan I, Gottesman MM. Modulation of activity of the promoter of the human *MDR*1 gene by Ras and p53. Science 1992;255:459-62.

Schoenlein PV, Shen D-w, Barrett JT, Pastan I, Gottesman MM. Double minute chromosomes carrying the human *MDR*1 and *MDR*2 genes are generated from the dimerization of submicroscopic circular DNAs in colchicine-seleceted KB carcinoma cells. Mol Biol of the Cell 1992;3:507-20.

Handler JS, Horio M, Willingham M, Pastan I, Gottesman MM. Polar localization of the multidrug resistance transporter in epithelia results in transepithelial transport of substrates. Amsterdam: Elsevier Science Publishers, 1992; in press.

Chin K-V, Chauhan SS, Abraham I, Sampson KE, Schimmer B, Wong M, Pastan I, Gottesman MM. Reduced mRNA levels for the multidrug-resistance genes in cAMP-dependent protein kinase mutant cell lines. J Cell Physiol 1992; in press.

Goldstein LJ, Pastan I, Gottesman MM. Multidrug resistance in human cancer. Critical Reviews in Oncology /Haematology1992; in press.

Mickisch GH, Rahman A, Pastan I, Gottesman MM. Liposome encapsulated doxorubicin is more effective in MDR transgenic mice than free doxorubicin. J Natl Cancer Inst 1992; in press.

Mickisch GH, Pai. LH, Gottesman MM, Pastan I. Monoclonal antibody MRK 16 reverses the multidrug resistance of MDR-transgenic mice. Cancer Res 1992;in press.

LeLong IH, Padmanabhan R, Lovelace E, Pastan I, Gottesman MM. ATP and GTP as alternative energy sources for vinblastine transport by P-170 in KB-V1 plasma membrane vesicles. FEBS Letters 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CB 08756-05 LMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Transgenic Mouse as a Model System to Study Gene Function and Regulation

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

PI:	G. T. Merlino	Expert	LMB,	NCI
Others:	I. Pastan	Chief, Laboratory of Molecular Biology		NCI
	M. M. Gottesman	Chief, Laboratory of Cell Biology		NCI
	C. Jhappan	Senior Staff Fellow	LMB,	NCI
	H. Takagi	Visiting Fellow	LMB,	NCI
	H. Takayama	Visiting Fellow	LMB,	NCI
	R. Sharp	Biologist	LMB,	NCI

COOPERATING UNITS *(if any)*

N. Fausto, Laboratory of Pathology & Laboratory Medicine, Brown University
G. Smith and R. Callahan, Laboratory of Tumor Immunology and Biology, DCBDC, NCI
D. Bockman, Dept. of Cellular Biology and Anatomy, Medical College of Georgia

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER.
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The advent of transgenic technology, whereby foreign DNA is stably introduced into the mammalian germ line, has provided a powerful means to study fundamental biological questions. We are continuing to use this technology to investigate the role of growth factors, receptors and oncogenes in the initiation and development of neoplasia, and to establish useful animal models to study the pathogenesis of human disease.

Both transforming growth factor α (TGFα) and epidermal growth factor (EGF) stimulate cellular proliferation by binding and activating the EGF receptor tyrosine kinase. Perturbation of the EGF receptor signal transduction pathway can transform cells in culture, and has been implicated in the development of human cancer. To test this hypothesis *in vivo*, transgenic mice were made bearing the human TGFα gene. TGFα overexpression was found to induce hepatocellular carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and a hypertrophic gastropathy resembling Menetrier's disease. Detailed molecular analysis of transgenic liver neoplasia has shown that TGFα promotes tumor formation and plays a role in tumor progression. Furthermore, other factors that may collaborate in TGFα-induced hepatocarcinogenesis have been found to include expression of *c-myc*, insulin-like growth factor II, sex hormones, and the genetic background upon which the transgene operates.

We have begun to generate transgenic mice containing foreign DNA encoding other interesting growth and differentiation factors. Transgenic mice made using an activated form of an EGF-related gene, *int-3*, which contains EGF repeats and is a member of the *Notch* gene family, develop severe hyperplastic lesions of multiple secretory glands and neoplasia of the salivary and mammary glands. The latter were also arrested in development and lactation deficient in all female *int-3* transgenic mice. In addition, male mice were sterile due to epididymal hyperplasia. These findings demonstrate *in vivo* that expression of the activated *Notch*-related *int-3* gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia.

Major Findings:

Transgenic mice are generated by microinjecting purified DNA fragments into the pronuclei of single-cell mouse embryos derived from inbred FVB/N mice. Surviving embryos are transferred into pseudopregnant CD1 foster mothers. Founder (F_0) transgenic pups are identified by Southern blot or polymerase chain reaction analysis of tail genomic DNA. Colonies of mice heterozygous for a transgene are established, and heterozygotes are frequently mated to produce homozygous animals. Transgene expression is assessed by Northern blot and RNase protection analysis of tissue RNAs.

We and others have shown that overexpression of the EGF receptor and its ligands (TGFα and EGF) can transform cells in culture. To determine the *in vivo* consequences of perturbing the EGF receptor signal transduction pathway, we have made transgenic mice using foreign TGFα and EGF receptor genes. In one series of experiments, a DNA fragment containing the human TGFα cDNA driven by the mouse metallothionein-1 promoter was microinjected into one-cell embryos. Mice bearing this transgene expressed human TGFα RNA and protein in the majority of tissues tested, including the liver, pancreas, stomach and breast. Elevated levels of TGFα were detected in the blood and urine of transgenic mice, as has been demonstrated in cancer patients. TGFα transgenic mice were characterized by the progressive development of a number of dramatic lesions, and will be useful as animal models for a number of important human diseases.

One year old male transgenic mice overexpression TGFα exhibited a high incidence (74%) of hepatocellular carcinoma. To identify factors associated with oncogenesis, liver tumors from transgenic animals were characterized at the molecular level. TGFα RNA transcripts were elevated in 17/25 (68%) liver tumors relative to adjacent nontumorous tissue. Expression of the endogenous *c-myc* and insulin-like growth factor II (IGF II) genes was enhanced in 7/19 (37%) and 12/16 (75%) liver tumors, respectively. In contrast, EGF receptor RNA levels were unchanged or reduced in all liver tumors, and point mutations were not detected in either the *Ha-ras* or *Ki-ras* gene. The occurrence of liver tumors in castrated TGFα transgenic mice was reduced about 7-fold, while in ovariectomized transgenic animals the incidence was increased about 6-fold. The progeny of a cross between CD1-derived TGFα transgenic (MT42) and C57BL/6 mice exhibited no reduction in tumor burden (83%); however, the incidence of tumor formation in MT42 X FVB/N offspring was substantially lower (19%). We conclude that in these transgenic mice TGFα promotes tumor formation, and appears to play a major role in tumor progression. Moreover, other factors that may collaborate in TGFα-induced hepatocarcinogenesis include expression of *c-myc*, IGF II, sex hormones, and the genetic background upon which the transgene operates. The collaboration of *c-myc* in MT42 liver oncogenesis was confirmed by mating our TGFα transgenic mice with another line of transgenic mice (made in the laboratory of Dr. Snorri Thorgeirsson) in which the *c-myc* transgene was overexpressed in the liver. Preliminary results indicate that dysplastic and malignant liver tissue arises at a much accelerated rate in the doubly transgenic animals.

TGFα transgenic mice also developed mammary adenocarcinomas in multiparous female mice. Nontumorous transgenic mammary tissue developed into cystic adenocarcinomas within 6 to 9 months when transplanted into nontransgenic syngeneic FVB host mammary fat pads. This result indicated that although autocrine or local paracrine TGFα stimulation of mammary tissue was responsible for triggering oncogenesis in the breast, other events were required as well. We also noted abnormal development of the mammary gland in TGFα transgenic mice.

TGFα also induced dramatic structural and functional lesions of the glandular stomach that resemble Menetrier's disease in humans. Transgenic mice developed 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 parietal cell function had been greatly impaired. These findings demonstrate that overproduction of TGFα can stimulate cellular proliferation, suppress acid secretion and perturb organogenesis of the stomach of transgenic mice. Moreover, TGFα may contribute to the pathogenesis of related human stomach disorders such as Menetrier's disease.

Finally, TGFα induced a florid ductular metaplasia of the pancreas, associated with severe interstitial fibrosis. The fibrosis caused the pancreas to become thick, hard and enlarged. The increase in size of the pancreas was found to be due to an increase in connective tissue, mainly type I collagen. Acinar cells appeared to redifferentiate into ductular cells and mucin-secreting cells, forming tubular complexes. The transgenic pancreas routinely displays properties that are characteristic of diseases of the human exocrine pancreas, including chronic pancreatitis. Although the two diseases differ in that there is no consistent chronic inflammatory response in the transgenic pancreas, the TGFα transgenic mouse may represent a valuable animal model to study the development and treatment of this human pancreatic disease.

In another series of experiments, transgenic mice were made bearing a DNA fragment containing the human EGF receptor cDNA driven by a chicken β-actin promoter. In one unique line of mice pronounced expression of the human EGF receptor was detected only in the testis. We were able to demonstrate that the transgene was being regulated by a translational control mechanism. Homozygous male mice were often found to be sterile due to sperm paralysis. Electron microscopic analysis of the tails of paralyzed sperm demonstrated that the axonemes were composed of an abnormal configuration of peripheral microtubules (5+2 instead of the normal 9+2). Axonemal aberrations of this type have been observed in the sperm of sterile men as well. This unique transgenic mouse line should provide a model for studying male sterility, spermatogenesis-specific gene expression, and translational control.

We are also using transgenic mice to study growth factors and receptors that are related to EGF. The gene encoding one such peptide (*int-3*) was identified by its role in mouse mammary gland oncogenesis. The mouse mammary tumor virus (MMTV) was shown in integrate into, and activate the *int-3* gene, which contains repeats similar to the yeast *cdc10* cell cycle start protein and multiple EGF repeats. We used a DNA fragment containing the activated *int-3* gene (under the transcriptional control of the MMTV long terminal repeat [LTR]) isolated from a mouse breast tumor to generate transgenic mice.

All six *int-3* founder transgenic mice and the progeny of one established line exhibited similar dramatic phenotypic abnormalities in all tissues in which the transgene was expressed, but not is nonexpressing tissues. Focal and often multiple poorly differentiated mammary and salivary adenocarcinomas appeared in the majority of transgenic mice between two and seven months of age. Mammary glands were arrested in development and lactation-deficient in all female *int-3* mice. Hyperplastic immature ductule cells appearing to be incompletely differentiated were observed in all salivary glands, glands of the nasal mucosa and maxillary sinus, the extraorbital lacrimal gland and the Harderian gland of juvenile and adult transgenic mice. In addition, all male transgenic founders were sterile due to severe hyperplasia of the epididymis. These findings demonstrate *in vivo* that expression of the activated *int-3* gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia. Doubly transgenic mice, made by mating TGFα and *int-3* transgenic mice, exhibited highly accelerated mammary gland tumorigenesis, suggesting that these two factors can function synergistically in the development of breast cancer in the mouse.

To determine the transcriptional mechanisms by which proto-oncogenes are regulated, we have now isolated and characterized the EGF receptor gene promoter using mutagenesis analysis, protein-binding assays, and a cell-free transcription system (see Annual Report Number Z01 CB 08000-22 for more details). To validate information obtained from transfection assays, and to study *in vivo* promoter function, fragments of genomic DNA containing all known EGF receptor gene *cis* elements required for optimal activity in transfected cultured cells were used to drive the expression of the chloramphenical acetyltransferase (CAT) reporter gene in transgenic mice. Unexpectedly, in all nine expressing lines of mice originally developed the EGF receptor gene promoter was most active in the thymus and to a lesser extent the spleen, based on quantification of CAT activity in a panel of transgenic mouse tissues. This selective expression has been confirmed by the generation and analysis of four additional transgenic lines using the same DNA construct. Crude cell separation techniques showed that the promoter was not active in thymocytes, but only in stromal tissue, containing thymic epithelial cells. More sophisticated techniques are presently being used to attempt to identify specific thymic epithelial cell types. Our results raise the possibility that EGF receptor may play a role in thymic epithelial cell function and/or thymocyte selection. We believe that this novel promoter will be useful in the future to target expression of various transgenes to the thymic epithelium.

In another project, we are continuing to collaborate with Drs. Gottesman and Pastan in the exploration of the utility of transgenic mice harboring a human gene (MDR1) encoding the 170 kDa P-glycoprotein. P-glycoprotein is capable of conferring multidrug resistance to animal cells, and when overexpressed allows malignant cells to evade destruction by chemotherapeutic agents (see Annual Report Number Z01 CB 08754-09 for details). Transgenic mice were generated in which the human MDR1 cDNA was expressed in the transgenic bone marrow, which in nontransgenic animals is exquisitely sensitive to chemotherapy. In these animals it was determined that the transgenic bone marrow was resistant to drug-induced leukopenia, and that bone marrow suppression was either greatly ameliorated or eliminated, depending on the specific drug and dosage. Furthermore, drug resistance in these transgenic mice could be defeated by the administration of appropriate reversing agents, such as verapamil. We are continuing to use these MDR1 transgenic mice as an *in vivo* model system to test the efficacy of novel chemotherapeutic drugs or combinations of drugs, including reversing agents which inhibit activity of the multidrug transporter.

Publications:

Merlino GT, Stahle C, Jhappan C, Linton R, Mahon KA, Willingham MC. Inactivation of a sperm motility gene by insertion of an EGF receptor transgene that is overexpressed and compartmentalized during spermatogenesis, Genes Dev 1991;5:1395-406.

Mickisch GH, Licht T, Merlino GT, Gottesman MM, Pastan I. Chemotherapy and chemosensitization of transgenic mice which express the human multidrug resistance gene in bone marrow: efficacy, potency and toxicity, Cancer Res 1991;51:5417-24.

Dickson, RB, Gottardis, MM, Merlino, GT. Molecular insights into breast cancer from transgenic mouse models. Bioessays 1991;13:1-6.

Gottesman MM, Horio M, Lelong I, Handler J, Raviv Y, Galski H, Mickisch G, Merlino G, Willingham M, Pastan I. Function of the multidrug transporter. In: Sugano H, Tsuruo T, eds. 13th Annual Bristol Myers Cancer Symposium on Drug Resistance as a Biochemical Target on Cancer Chemotherapy, Vol 9. New York: Academic Press, 1991;45-62.

Merlino GT, Mickisch G, Jhappan C, Galski H, Takagi H, Smith GH, Fausto N, Pastan I, Gottesman MM. Cellular proliferation and drug resistance in transgenic mice. In: Verna, R, Nishizuka Y, eds. Biotechnology of Cell Regulation. Raven Press: New York, 1991;271-83.

Mickisch GH, Merlino GT, Alken PM, Gottesman MM, Pastan I. New potent verapamil derivatives that reverse multidrug resistance in human renal carcinoma cells and in transgenic mice expressing the human MDR1 gene, J. Urology 1991;146:447-53.

Merlino GT. Transgenic animals in biomedical research, FASEB J. 1991;5:2996-3001.

Jhappan C, Gallahan D, Stahle C, Chu E, Smith GH, Merlino G, Callahan R. Expression of an activated *Notch*-related *int-3* transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands, Genes Dev 1992;6:345-55.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08757-05 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Development of Immunotoxins for Cancer

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

PI:	D. J. FitzGerald	Microbiologist	LMB,	NCI
Others:	I. Pastan	Chief, Laboratory of Molecular Biology,		NCI
	M. Chiron	Visiting Fellow	LMB,	NCI
	A. Zandovsky	Visiting Fellow	LMB,	NCI
	C. Fryling	Biologist	LMB,	NCI
	M. Thompson	Special Volunteer	LMB,	NCI

COOPERATING UNITS *(if any)*

C. B. Saelinger, Dept. of Microbiology and Molecular Genetics, University of Cincinnati Medical School, Cincinnati, Ohio; D. Strickland, American Red Cross, Rockville, Maryland

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.2	4.2	1.0

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.)*

PE binds and enters mammalian cells and is then processed to an active C-terminal fragment which translocates to the cell cytoplasm and inhibits protein synthesis by ADP-ribosylating EF-2. At the cell surface, PE binds to a large MW glycoprotein which has been identified as the α_2-macroglobulin receptor (α_2M-R). In an ELISA assay PE but not PEGlu57 bound to affinity purified α_2M-R. A 39 kD receptor associated protein (RAP) blocked PE binding to intact cells and pretreatment of cells with RAP prevented toxin-mediated inhibition of protein synthesis. Once delivered to the endosomal compartment by the α_2M-R, PE is cleaved by an membrane-associated protease (MAP). Purification of this protease has begun using beef liver as a source of the enzyme. Crude membranes are treated with papain to release the MAP in a soluble form. Column chromatography is used to complete the purification of the soluble enzyme. In the endosome PE is cleaved between arginine 279 and glycine 280 and then reduced to generate a 37 kD C-terminal fragment which translocates to the cytosol. In the presence of excess PE553D (a mutant form of PE lacking ADP-ribosylating activity), the toxicity of native PE is greatly reduced. However, excess PE553D does not compete strongly for the binding of native PE to its surface receptor, rather it competes within cells for a step taken by the 37 kD fragment *en route* to the cytosol. Mutant forms of PE553D with substitutions of other amino acids for Trp281 compete dramatically less well than PE5553D for this step. PE553D with an alanine in place of tryptophan 281 competes 100-fold less well than PE553D, despite the fact that PE553DAla281 binds to cells, is internalized, and is cleaved appropriately by the cellular protease. In PE, glycine 280 can be changed to methionine without loss of biological activity. This allows the production of recombinant forms of PE that begin at residue 280 (with methionine in this position) and do not need to be processed within cells. PE37-Transforming Growth Factor-alpha (TGFα) which was constructed by cloning TGFα into the C-terminal end of PE37 was found to be more active than TGFα-PE40, possibly because there was no need for cellular processing.

PHS 6040 (Rev. 1/84)

GPO 914-918

Major Findings:

Characterization of the Cell Surface Receptor for PE:
Pseudomonas exotoxin (PE) kills multiple cell types isolated from many different species. To be broadly effective as a virulence factor, PE must take advantage of molecules which are highly conserved in nature. The α_2-macroglobulin receptor (α_2-MR), which is widely distributed and highly conserved, is a large cell-surface glycoprotein consisting of a 515 kDa and an 85 kDa polypeptide and is thought to be responsible for the binding and endocytosis of activated α_2-macroglobulin and *apoE*-enriched β-VLDL. A similar high molecular weight glycoprotein has been identified as a potential receptor for PE (Thompson *et al., J. Biol. Chem.* 266:2390-6, 1991). Upon SDS-PAGE human α_2-MR, that had been affinity purified on immobilized α_2-M, and the PE binding glycoprotein, which had been affinity purified on a PE column, had identical mobilities. By immunoblot analysis, rabbit antibodies raised to the human α_2-MR cross reacted with murine PE receptor protein. Likewise antibodies to the PE receptor recognized the human α_2-MR. Thus by size and immunological analysis both receptors appeared to be related, if not identical. To test for functional relatedness, an ELISA assay was used to show that affinity purified α_2-MR binds specifically to PE, but not to PEGlu57, a mutant toxin defective in its ability to bind cells. Associated with the α_2-MR is a 39 kD protein (designated RAP) that is thought to regulate ligand binding. The influence of this protein on PE binding and toxicity was also investigated. Pretreatment of cells with RAP blocked PE binding and also prevented toxin mediated inhibition of protein synthesis. The concentration of RAP that was required to reduce binding and toxicity to 50% was approximately 14 nM, a value virtually identical to the kD measured for the interaction of RAP with the purified receptor. Further, the specificity of the RAP effect on PE binding was confirmed by showing that RAP had no effect on the toxicity of TF-PE40 (a conjugate between human transferrin and domains II and III of PE), a conjugate that bound and entered cells on another receptor. Despite the fact that RAP blocked the binding and toxicity of PE and also blocks the binding of α_2-M to its receptor, it was not possible to show that α_2-M and PE competed with each other for binding to purified receptor. Nor did pretreatment of cells with excess α_2-M reduce PE-mediated toxicity. This raises the possibility that RAP can regulate ligand binding without occupying the ligand binding site, *e.g.* RAP could cause a conformation change in the receptor that reduces the availability of ligand binding sites. Overall, the studies strongly suggest that the α_2-MR is responsible for binding and internalizing PE.

Determination of the Site of Proteolytic Cleavage Within PE:
Pseudomonas exotoxin (PE) is cleaved by a cellular protease within cells and then reduced to generate two prominent fragments (Ogata *et al., J. Biol.Chem.* 265:20678-85, 1990). The N-terminal fragment is 28 kD in size and contains the binding domain. The 37 kD C-terminal fragment, which translocates to the cytosol, contains the translocation domain and the ADP-ribosylation domain. Cleavage followed by reduction is essential for toxicity since mutant forms of the toxin that cannot be cleaved by cells are non toxic. Previous results with mutant proteins suggested that cleavage occurred in an arginine-rich disulfide loop near the beginning of the translocation domain but the exact site of cleavage was not determined. Since very few molecules of the 37 kD fragment are generated within cells it was not possible to determine the site of cleavage by performing a conventional N-terminal sequence analysis of the 37 kD fragment. Two methods have been employed to determine the N-terminal sequence of the 37 kD fragment. Both involve producing intrinsically labeled toxin (or toxin mutants) by growing *E. coli*, harboring the appropriate plasmid, in minimal media containing a radioactive amino acid. The first method uses 35S-methionine labeled PE, which is added to mammalian cells and later the processed fragments are immunoprecipitated. Since all naturally occurring methionines are in domain I (Gray, G.L.*et al.*: *Proc. Natl. Acad. Sci. USA*. 81:2645-49, 1984), the N-terminal 28 kD fragment but not the 37

kD fragment is immunoprecipitated from cells as a radiolabeled fragment (Ogata *et al., J. Biol. Chem.* 265:20678-85, 1990). Various mutants have been constructed whereby amino acids in the immediate vicinity of the arginine-rich loop were changed to methionine residues. Placement of a methionine residue at the N-terminal side of the cleavage site should produce a mutant toxin that, when added to cells, is cleaved to produce a radioactive 28 kD fragment and a nonradioactive 37 kD fragment. However, placement at the C-terminal side of the cleavage site should produce a mutant that when added to cells produces both a radioactive 28 and 37 kD fragment. Results indicate that when methionine was substituted for glutamine at position 277, a fully toxic molecule was produced. When 35S-methionine-labeled PEmet277 was added to cells, only the 28 kD fragment was recovered as a radioactive fragment. When methionine was substituted for glycine at 280, a fully active molecule was also produced. Immunoprecipitation of PEmet280 from cells recovered both fragments as radiolabeled products. This indicated that cleavage was occurring after 277 and before 280. Substitution of methionine for proline at 278 produced a molecule PEmet278 that had reduced toxicity and was less susceptible to cell mediated proteolysis. After immunoprecipitation, a small amount of radiolabeled 28 kD fragment but no labeled 37 kD was recovered. This result indicated that proline is important in making PE a suitable substrate for proteolysis and that cleavage probably occurred after residue 278. Since no proteolysis occurs when arginine 279 is replaced by other residues, this kind of analysis cannot be used to distinguish between cleavage that occurs immediately before or after this residue.

The methionine substitutions provided additional kinds of information. First, the substitution of residues 280-284 with methionines did not interfere with proteolysis. Thus the critical residues interacting with the protease were located at the N-terminal and not C-terminal side of the cleavage site. Second, the replacement of tryptophan 281 with methionine produced a molecule that was cleaved but was nontoxic. The role of tryptophan 281 is discussed below. Third, the replacement of glycine 280 with methionine generated a fully toxic molecule. If cleavage occurs after arginine 279, then the N-terminus of the 37 kD would normally be glycine but could also be methionine. Thus one could make the 37 kD fragment by recombinant means by constructing a truncated toxin gene beginning with AUG and then continuing with codons that code for amino acids 281-613. Such a construction has been made and the possibilities for making recombinant chimeric toxins is discussed below.

As mentioned above two methods have been employed to determine the N-terminal sequence of the 37 kD fragment. The second also uses intrinsically labeled toxin. Transformed host cells are grown in media containing ^{3}H-leucine, the toxin is purified from the periplasm and radioactive toxin added to cell. The 37 kD fragment is then recovered from cells by immunoprecipitation and separated from antibody and other fragments by SDS-PAGE. The proteins are transferred to Immobilon membranes and the 37 kD fragment cut out and sent for sequence analysis. Analysis involves the collection of fractions after each round of Edman degradation. Each fraction is then counted in a scintillation counter and fractions containing radioactivity above background indicate the location of leucine residues. This kind of analysis revealed that the amino acid after the fifth Edman cycle was a leucine. This result confirmed that cleavage occurred between the arginine at residue 279 and the glycine residue at 280.

Role of Tryptophan 281 in PE toxicity:
As mentioned, PE is proteolytically cleaved within cells between arginine 279 and glycine 280 and is then reduced to generate a 37 kD C-terminal fragment which translocates to the cytosol and ADP-ribosylates elongation factor 2. The second amino acid from the N-terminus of the 37 kD C-terminal fragment is tryptophan. When this amino acid was changed to methionine, there was a reduction of PE toxicity by at least 100-fold. To determine the role of tryptophan at this position several other mutations were generated.

Substitution of trytophan by alanine, serine, phenylalanine, histidine, glutamic acid, arginine, and lysine all produced mutant forms of PE that were less active than wild type PE. PEW281K was five-fold less active, PEW281R and PEW281H were 30-fold and the others were at least 100-fold less active. Since the mutations were close to the proteolytic cleavage site, selected mutants were tested for their susceptibility to cell-mediated proteolysis. PEW281R and PEW281A were each cleaved more efficiently than native PE. The resulting C-terminal 37 kD fragment produced from PEW281A was sequenced and found to begin with glycine 280. Thus substitutions, rather than interfering with cell-mediated proteolysis, seemed to favor it. To investigate the role of these amino acids at this residue position, further experiments were carried out.

When excess PE553D (a mutant form of PE lacking ADP-ribosylating activity) was added to cells, the toxicity of native PE was greatly reduced. However, excess PE553D did not compete strongly for the binding of native PE to its cell surface receptor, rather it competed within cells for a step taken by the 37 kD fragment *en route* to the cytosol. Mutant forms of PE553D with substitutions of other amino acids for tryptophan 281 competed dramatically less well than PE5553D for this step. PE553D with an alanine in place of tryptophan 281 competed 100-fold less well than PE553D, despite the fact that PE553DAla281 binds to cells, is internalized, and is cleaved appropriately by the cellular protease. Competition takes place after the cleavage step since mutant forms of PE that cannot be cleaved by cells (e.g PEgly276) do not compete for toxicity despite having a tryptophan residue at position 281. Attempts are currently underway to identify the cellular component that interacts with tryptophan 281.

Purification of the Cellular Protease that Cleaves PE:
Initial attempts at the purification of the protease that cleaves PE were not successful. Purification from tissue culture was not feasible and detergent extracts of liver membranes produced a crude protease whose activity spread broadly on various chromatography columns. However, releasing the protease by papain digestion of crude liver membranes has lead to a 500-fold purification (to date) of the protease and much "cleaner" separations using chromatographic techniques. Purification to homogeneity, or close enough to get amino acid sequence data, continues to be a major focus of the laboratory.

Publications:

Siegall CB, Kreitman RJ, FitzGerald DJ, Pastan I. Antitumor effects of interleukin 6-*Pseudomonas* exotoxin chimeric molecules against the muan hepatocellular carcinoma, PLC/PRF/5 in mice, Cancer Res 1991;51:2831-6.

Siegall CB, Ogata M, Pastan I, FitzGerald DJ. Analysis of sequences in domain II of *Pseudomonas* exotoxin A which mediate translocation, Biochem 1991;51:7154-9.

Pastan I, FitzGerald D. Recombinant toxins for cancer treatment, Science 1991;254:1173-7.

FitzGerald D, Pastan I. Redirecting Pseudomonas exotoxin, Seminars Cell Biol 1991;2:31-7.

Pai LH, Bookman MA, Ozols RF, Young RC, Smith JW, Longo DL, Gould B, Frankel A, McClay EF, Howell S, Reed E, Willingham MC, FitzGerald DJ, Pastan I. Clinical evaulation of intraperitoneal *Pseudomonas* exotoxin immunoconjugate OVB-3PE in patients with ovarian cancer, J Clin Oncol 1991;9:2095-103.

Fattom A, Shiloach J, Bryla D, FitzGerald D, Pastan I, Karakawa WW, Robbins JB, Schneerson R. Comparative immunogenicity of conjugates composed of the *Staphylococuss aureus* type 8 capsular polysaccharide bound to carrier proteins by adipic acid dihydrazide or N-succinimidyl-3-(2-pyridyldithio)propionate, Infec & Immun 1992;60:584-9.

Fryling C, Ogata M, FitzGerald D. Characterization of a cellular protease that cleaves *Pseudomonas* exotoxin, Infec & Immun 1992;60:497-502.

Prior TTI, FitzGerald DJ, Pastan I. Translocation mediated by domain II of *Pseudomonas* exotoxin A: Transport of barnase into the cytosol, Biochem 1992;31:3555-9.

Kreitman RJ, FitzGerald D, Pastan I. Targeting growth receptors with fusion toxins, Int J Immunopharmac 1992;14:465-72.

Kounnas MZ, Morris RE, Thompson MR, FitzGerald DJ, Strickland DK, Saelinger CB. The α_2 macroglopubin receptor/LDL receptor related protein binds and internalizes *Pseudomonas* exotoxin A, J Biol Chem 1992; in press.

Theuer CP, FitzGerald D, Pastan I. A recombinant form of *Pseudomonas* extoxoin directed at the epidermal growth factor receptor that is cytotoxic without requiring proteolytic processing, J Biol Chem 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08758-01 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Transcription Mechanism and Structure-function Analysis of RNA Polymerase of *E. coli*

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

PI:	D. J. Jin	Senior Staff Fellow, DGS	LMB,	NCI
Other:	M. Xu	Special Volunteer	LMB,	NCI
	Y. Yee	Special Volunteer	LMB,	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Developmental Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-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 overall goal of this study is to dissect the catalytic center and the regulatory domain(s) of RNA polymerase of *Escherichia coli* to study the mechanisms of transcription. In particular, the effect on different steps of transcription of mutations in RNA polymerase that confer rifampicin resistance (rif^r) have been analyzed. The antibiotic rifampicin inhibits transcription of RNA polymerase from all eubacteria indicating that the rifampicin binding site(s) is in a region that may be conserved and important for RNA polymerase functions. RNA polymerases with altered rifampicin binding are likely to be also altered in some vital transcription processes. By studying the correlation between a particular rif^r RNA polymerase and its altered transcription property, one can assign the functional role of the rifampicin binding site(s) of RNA polymerase.

Some of rif^r RNA polymerases are found to be defective in promoter clearance (a transition step in transcription at which an RNA polymerase exits from the initiation stage to the elongation stage) and to enhance abortive initiation products suggesting that the rifampicin binding site(s) is involved in this important process. The mechanisms underlying the defect in promoter clearance of one rif^r RNA polymerase in which there is an Arg 529 Cys change in the β subunit have been studied in detail both *in vivo* and *in vitro*. This mutant RNA polymerase is found to have reduced affinity for a nucleoside triphosphates during initiation and is subject to a high Km barrier during promoter clearance process. Since this rif^r RNA polymerase also has reduced affinity for the same nucleoside triphosphates during elongation, it is plausible that the amino acid residue Arg 529 of the β subunit is part of the catalytic center of RNA polymerase.

Major Findings:

Effect of rif^r RNA polymerases on initiation from the *pyrBI* promoter:

1. In addition to being subject to UTP-sensitive attenuation control, the pyrimidine biosynthetic operon *pyrBI* is controlled at the initiation step by the availability of UTP. Since rifampicin inhibits transcription initiation, it is reasonable to expect that mutations that alter the rifampicin binding of some rif^r RNA polymerase might change the property of initiation at the *pyrBI* promoter. In collaboration with Dr. Charles L. Turnbough, Jr. at the Department of Microbiology, University of Alabama, Birmingham, the effects of different rif^r RNA polymerase on the initiation of the *pyrBI* promoter were analyzed. We have found that six out of twelve rif^r RNA polymerase have increased abortive initiation products compared to the wild type RNA polymerase. The result suggests that part of the rifampicin binding site(s) defined by the rif^r mutations is involved in promoter clearance.

2. Of the six rif^r RNA polymerases that increased abortive initiation products, RpoB3401 is the most defective one. We have found that the promoter clearance defect of RpoB3401 is sensitive to UTP concentration: the lower UTP concentration, the more severe the defect. The defect of RpoB3401 is not corrected when 5' BrUTP is used in the place of UTP. These results not only indicate that RpoB3401 has a reduced affinity for UTP during promoter clearance but also suggest that, in general, a high Km barrier can be a limiting step for an initiating RNA polymerase to make the transition to elongation.

3. Using a *pyrBI::lacZ* fusion which acts as a reporter of initiation alone, we have found that the *rpoB3401* allele reduces *lacZ* expression about 10 to 20-fold relative to the *rpoB*$^+$ isogenic strain. This *in vivo* result is consistent with the *in vitro* results confirming that the mutant RNA polymerase is defective in initiation.

4. RpoB3401 has a slower rate of elongation than the wild type enzyme on a *pyrBI* template and enhances pausing at the UTP-sensitive pausing sites. The elongation defect can be overcome by merely increasing the UTP concentration. This result indicates that RpoB3401 also reduced affinity for UTP during elongation. Since a mutation change a residue 529 (Arg to Cys) of the β subunit of RNA polymerase alters both the initiating and elongating Km for UTP it is plausible that this residue is involved in the catalytic center of RNA polymerase.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08759-01 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Molecular Modeling

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

PI:	B. K Lee	Chief, Molecular Modeling Section,	LMB,	NCI
Other:	J. R. Kim	Visiting Fellow,	LMB,	NCI
	N. Kurochikina	Visiting Fellow	LMB,	NCI
	S.-H. Jung	Visiting Fellow	LMB,	NCI
	J. Cammisa	Computer Specialist	LMB,	NCI

COOPERATING UNITS *(if any)*

Molecular Biology Section, LMB, DCBDC, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Modeling Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	3.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The main research interest of the Section is to use various theoretical means to study the forces that govern the structure and interaction of globular protein molecules, to predict the three-dimensional structure of these molecules, and to engineer protein molecules with improved properties. Following were accomplished: (1) The molecular graphics modeling program, GEMM, has been improved; (2) The origin of the hydrophobic effect has been investigated and its magnitude has been estimated relative to the non-hydrophobic effect on the stability of globular proteins; (3) Two different approaches have been devised to predicting the three-dimensional structure of a globular protein from its amino acid sequence alone and the strength and the shortcomings of each approach have been evaluated.

Major Findings:

A. Computer graphics (with Dr. J. R. Kim and Mr. J. Cammisa)

All modeling work requires a good computer molecular graphics program in order to view the molecules conveniently. The Section has a home-grown molecular graphics program, GEMM, for this purpose. During the past year, we have identified and corrected a number of bugs in the program. A few enhancements have also been incorporated. These include the ability to turn on and off the display of specific side chains, ability to read and write coordinate files in the format of the molecular dynamics program, CHARMM, and an ability to randomly change the dihedral angles for a specified range of residues. These changes are generally useful for all modeling work, but particularly useful in modeling molecules that have a small number of mutations and other alterations. The program was used to produce a number of images of chimeric toxin molecules for the researchers in the Molecular Biology Section.

B. Hydrophobic effect and protein stability

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

The hydrophobic effect is most directly measured by the change in the free energy, ΔG, when a small non-polar molecule is transferred from a non-polar medium to water. This change arises from the change in the direct interaction, ΔEa, between the solute molecule with the two different types of the solvent molecules and from the different contributions from the effect of solvent reorganization, ΔGr, that occur when a foreign molecule is introduced to a solvent. Of these two terms, ΔEa has simple physical basis and can be reliably estimated from computer simulation studies. Most controversy, therefore, centers on the origin, magnitude, and temperature dependence of the second term. Last year, we unambiguously determined the magnitude of this term at a range of temperatures by subtracting ΔEa obtained from the computer simulation studies from the experimentally obtained values for ΔG. It was then found that the resultant values for ΔGr agree excellently with a simple theory that treats all solvent molecules as isotropic spheres. This result indicates that the origin of ΔGr term and therefore of the hydrophobicity is in the small size rather than the hydrogen bonding property of water molecules.

Application of this effect to the protein stability is complicated because: (1) there are many forces other than the hydrophobic effect that operate when a protein unfolds; and (2) the protein interior is a special environment, possibly significantly different from the a non-polar liquid which is used to determine the hydrophobic effect in small molecule transfer experiments. The experimental thermodynamic data on protein unfolding show a peculiar property: when the specific enthalpy changes upon unfolding different proteins are plotted against temperature, they were found to converge at about 112°C, i.e. at this temperature, all proteins unfold with

same specific enthalpy change. Similar behavior is observed for the specific entropy changes. All theories on protein stability must explain this peculiar feature of the protein unfolding thermodynamics. Last year an analysis was made of these data in conjunction with what is known about the hydrophobic effect as it applies to the small molecule transfer experiments. This study indicated that: (1) the polar and non-polar effects are about equally important in stabilizing the tertiary structure of a protein molecule and (2) when protein unfolds, its water contact surface area increases approximately by a factor of two, which is considerably smaller than what is expected if all parts of the unfolded form were completely exposed to water. These findings have practical importance in the design of a successful protein folding program.

C. Protein folding (with Dr. J. R. Kim and Dr. N. Kurochikina)

Compared to the number of proteins whose sequence is known, the number of proteins for which the structure is known is appallingly small. The need for a technique for obtaining the three-dimensional structure of a protein molecule from its amino acid sequence has perhaps never been greater. The goal of this project is to develop an effective technique for predicting the three-dimensional structure of a globular protein molecule from its amino sequence alone. This is a difficult problem that requires a long term commitment with no prospect of frequent publications.

We worked on this problem using two different approaches. In one approach, the neural network (NN) programming technique was used to predict the secondary structure. By using two sets of NNs in two stages, we were able: (1) to classify protein structures with 71% accuracy; and (2) to use this information to predict the secondary structures with 68%, 68%, and 62% accuracy for the α-rich, β-rich, and the mixed class proteins, respectively. These numbers indicate that our program performs better than any that have been reported for this purpose. On the α-class proteins, in particular, the improvement over the best reported technique is nearly 10%. Currently, attempt is being made to use this result of the secondary structure prediction to predict the tertiary structure using genetic algorithm.

In another approach, massive numbers of different conformations are sampled using a biased Monte Carlo procedure. A large amount of information from known protein structures is incorporated into a set of probability tables for the mainchain dihedral angles, which are then used as the bias in the Monte Carlo sampling step. We found that these probability tables can be used to compute a function akin to the entropy change upon folding. The values of this function, when plotted along the sequence of a protein, were found to correlate well with the pattern of early protection against hydrogen exchange in test tube protein folding experiments. This function can, therefore, be used to predict early folding intermediates. The quality of the three-dimensional structure one obtains by the Monte Carlo procedure depends on the potential energy function used. Using empirical residue pair potential of Miyazawa and Jernigan, one obtains structures that are topologically correct but deviate from the crystal structure in detail. Augmenting the potential energy by inclusion of hydrogen bonds improves detail but tends to form structures that have too long helices. The overall deviation from the x-ray structure in the case of the small plant protein crambin is about 4 A (in d-rms). Additional improvement is not expected without incorporating the disulfide bond formation. Handling disulfide bond is difficult because dihedral angle rotations tend to break these bonds. Future effort will be concentrated on devising moves that preserve disulfide bonds and on improving the potential energy function.

D. Modeling with *Pseudomonas* exotoxin system (with Dr. S.-H. Jung)

In collaboration with the Molecular Biology Section, we are attempting to improve the properties of various chimeric molecules derived from the *Pseudomonas* exotoxin (PE). This is a new project that we have just begun when Dr. Jung joined our laboratory at the end of March. We have finished two short projects so far: (1) A model structure of the Fv portion of the anti-tumor antibody B3 was produced by mutating the crystal structure of the phosphocholine binding antibody, M603. This molecule will be used in future studies into the property of various B3-PE conjugate molecules; (2) Searched for sequence homology between domain II of PE and the heat labile entrotoxin from *E. coli* and other sequences in the sequence data base. This search turned up nothing except for a weak homology with two transmembrane proteins. This weak homology could be important, but since neither of the transmembrane protein structure is known, the information is useless at this stage.

We have obtained a copy of the computer program DELPHI, which computes the electrostatic interaction energy, and are in the process of installing the program. The commercial software QUANTA and its subcomponent HELIX have been purchased and are being installed also. These programs will be used, in conjunction with other programs that already exist in the laboratory, to study the stability of the Fv portion of immunoglobulins.

Publications:

Lee B. Isoenthalpic and isoentropic temperatures and the thermodynamics of protein denaturation,.Proc. Natl. Acad. Sci. USA, 1991;88:5154-5158.

Lee B. Solvent reorganization contribution to the transfer thermodynamics of small non-polar molecules, Biopolymers, 1991;31:993-1008.

LABORATORY OF CELL BIOLOGY

SUMMARY REPORT

DCBDC, NCI

October 1, 1991 to September 30, 1992

The Laboratory of Cell Biology is composed of Molecular Cell Genetics Section (Michael M. Gottesman, Chief) and the Chemistry Section (Ettore Appella, Chief). The Chief of the Laboratory of Cell Biology supervises research emphasizing the molecular basis of drug resistance in cancer cells, the molecular basis of suppression of malignant transformation, the biological role and mechanism of ATP-dependent and acid proteases, the process of melanogenesis, and the mechanism of antigen processing. Approximately 32 personnel working on 7 specific research projects have contributed to the progress outlined in this summary.

Resistance of Cancer Cells to Anti-Cancer Drugs

It is possible to mimic the intrinsic and acquired resistance of human cancers to chemotherapy by selecting for resistance of cultured human cancer cells to specific drugs. Work in the Molecular Cell Genetics Section of the Laboratory of Cell Biology in collaboration with the Laboratory of Molecular Biology (Ira Pastan, Chief) has emphasized studies on resistance to natural product anti-cancer drugs (e.g., anthracyclines, vinca alkaloids, and etoposide) and cis-platinum. The major mechanism of resistance to natural product drugs is expression of the *MDR*1 gene which encodes the 170,000 dalton P-glycoprotein, an energy-dependent multidrug efflux pump. Analysis of this multidrug transporter through photoaffinity labelling studies, mutational alterations, and kinetic studies of drug transport, has led to a model in which natural product hydrophobic drugs are removed directly from the plasma membrane. Two general strategies are being explored to exploit our knowledge of the multidrug transporter to improve chemotherapy. In the first approach, agents which inhibit the activity of the multidrug transporter have been studied in an *MDR*1 transgenic mouse system. In the second approach, retroviral vectors encoding the *MDR*1 gene are being used to confer a multidrug-resistant phenotype on chemotherapy-sensitive tissues such as bone marrow to allow dose intensification of anti-cancer drugs.

Suppression of growth of malignant cells

Both cAMP and p53 are able to slow the growth of certain malignant cells. Work by Michael Gottesman in the Molecular Cell Genetics Section has focused on the molecular basis for activation of cAMP dependent protein kinase by cAMP. Mutations in cAMP dependent protein kinase have been selected directly in tissue culture cells which grow in the presence of cAMP analogs or agents which increase intracellular cAMP levels. Cloned regulatory and catalytic subunits of cAMP dependent protein kinase have been mutagenized *in vitro* to obtain novel mutants of these kinase subunits which fail to respond to cAMP stimulation both in mammalian and bacterial cells. Since some of these mutants have dominant

negative effects when expressed in mammalian cells, it is possible to create moveable genetic elements which ablate cAMP responses in recipient cells.

Stephen Ullrich in the Chemistry Section has found that the anti-proliferative effect of the tumor suppressor gene, p53, inhibits the expression of several late G1 growth regulated genes including *b-myb*, PCNA, and DNA polymerase α. Since *b-myb* probably functions as a transcription factor required for G1 progression, cell cycle arrest by wild-type p53 may be primarily the result of inhibition of *b-myb* -dependent transcription. Growth arrest mediated by wild-type p53 is also associated with increased phosphorylation of its amino-terminus compared to mutant p53. This phosphorylation has minimal effect on the anti-proliferative function of p53, but affects the intracellular amounts of p53 by decreasing the half-life of p53.

Proteolysis

In the Molecular Cell Genetics Section, Michael Maurizi has been studying the mechanism of energy utilization and selection of substrates by ATP-dependent proteases. Mutagenesis of the ATP-binding sites in the regulatory component of Clp protease has demonstrated the dual roles of ATP in allosterically activating Clp protease and in promoting changes in the substrate/protease interaction to facilitate processive cleavage of large proteins. Consistent with the above results, chemically synthesized peptide analogs of *in vivo* substrates have been shown to be specifically cleaved at a single site by Clp in a reaction that requires ATP binding but not hydrolysis. Work with another ATP-dependent protease, Lon, has resulted in the characterization of a phage-encoded inhibitor that binds with very high affinity to the protease.

Michael Gottesman has continued studies on cathepsin L, an acid lysosomal protease which is overproduced and secreted by malignantly transformed mouse and human cells. The human cathepsin L gene has been isolated and it appears to have two promoters; the downstream promoter is in the first intron for the gene expressed from the upstream promoter. A deletion analysis of the cathepsin L cDNA shows that secretion of cathepsin L requires an intact carboxy-terminus.

Melanoma and Melanogenesis

Vincent Hearing has studied the normal regulatory controls of melanogenesis and the expression of surface antigens by melanoma cells which affect their immunogenicity. Multiple loci have now been cloned which encode tyrosinase-related proteins; each of those has distinct catalytic properties which affect the quantity and quality of the melanins produced. These proteins interact in a melanosomal complex to synergistically regulate melanogenesis, and are believed to affect the structure and function of the pigment (e.g., its photoprotective and cytotoxic properties). These studies have potential importance due to the essential nature of melanin in protecting against the carcinogenic effect of UV radiation. The B700 melanoma surface antigen, an albuminoid antigen specifically produced by mammalian melanomas, is also being characterized. Monoclonal antibodies have been produced that specifically recognize the B700 antigen and have now been successfully used in antimetastatic protocols to treat pulmonary metastases in mice. In view of their specific cross-reactivity with human melanomas, these antibodies are being developed as potential probes for immunoassay and/or immunotherapy of human melanomas.

Antigen Processing

Research by Ettore Appella in the Chemistry Section has utilized the technique of microcapillary high-performance liquid chromatography/tandem mass spectrometry to fractionate and sequence subpicolar quantities of natural peptides bound to class I and class II MHC molecules. The sequence of eight different peptides bound to human class I MHC HLA-A2.1 and nine peptides associated with the MHC class II I-A^d has been determined. Several of these sequences could be identified in normal cellular proteins. Peptides extracted from HLA-A2.1 expressed on the mutant T2 cell line were derived from the signal peptide domains of normal cellular proteins. These peptides contain more than nine residues. These results suggest that proteolysis of signal peptide domains in the endoplasmic reticulum is an alternate mechanism for processing peptides for association with class I molecules. In the mutant T2 cell line, the production of antigenic peptides and/or the intracellular formation of complexes with class II molecules appears to be impaired. The predominance of invariant chain-derived peptides associated with the class II molecules produced in the T2 mutant suggests the possibility that the production of most peptides that ordinarily associate with class II molecules occurs in a cellular compartment different from the one in which the observed invariant derived peptides were produced. These recent observations provide the first evidence that more than one pathway is operating for producing class II/peptide complexes.

Additional studies in the Chemistry Section have highlighted the role played by a nuclear binding protein in mediating interferon response in cells of the immune system. Further work has been carried out toward the evaluation of the immunogenicity of a novel antigen, gp110 in *in vitro*-based immunological assays and the analysis of the structure of peptides bound to class I molecules on the surface of mouse methylcholanthrene tumors. These studies should define the molecular basis for the antigenic diversity associated with these tumors. Finally, the structure of a cellular transcription factor, important as a control element of transcription of the HIV-1 virus, has been determined and experiments are being carried out with a nucleocapsid protein of 71 amino acids of HIV-1 to assess its binding properties to RNA and unravel the various stages of viral replication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03229-22 LCBGY

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

T-Cell Antigen Recognition and Tumor Antigens

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

E. Appella	Medical Officer	LCB, DCBDC, NCI
K. Sakaguchi	Visiting Fellow	LCB, DCBDC, NCI
S.J. Ullrich	Expert	LCB, DCBDC, NCI
M. Fiscella	Visiting Fellow	LCB, DCBDC, NCI
N. Zambrano	Visiting Fellow	LCB, DCBDC, NCI
K. Ozato	Research Microbiologist	LDMI, NICHD
A. Gronenborn	Research Chemist	LCP, NIDDK

COOPERATING UNITS *(if any)*

Cytel, La Jolla, CA; Preclinical Research, Sandoz Ltd., Basel, Switzerland; Mikrobiologisk Institute, Copenhagen, Denmark; A.R.C., Villejuif, France

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Chemistry

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
5	5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Our laboratory is analyzing the structure of peptides bound to both class I and class II major histocompatibility (MHC) molecules. Micro capillary high-performance liquid chromatography/tandem mass spectometry was used to fractionate and sequence subpicomolar amounts of peptides. The peptides bound to class I MHC molecules HLA-A2.1 all were nine residues long, and contained LEU/ILE at position 2, a hydrophobic residue at position 1 and 8 and hydrophobic alkyl side chains at position 9. This motif is entirely different from that of peptides bound to HLA-B27. Possible precursor proteins for three of the peptides were identified and all three proteins have an intracellular location. Peptides presented by class II MHC molecules are typically 14-19 residues in length and exhibit ragged N- and C-termini. Those analyzed to date are all derived from secretory or integral membrane proteins and contained a six residue binding motif that is variably placed within the peptide chain. HLA-A2.1 peptides extracted from cells of the antigen processing mutant T2 were derived from the signal peptide domains of normal cellular proteins. These results suggest that there is a second antigen processing pathway for presentation of peptides by class I molecules in the endoplasmic reticulum. DR3 class II peptides extracted from the mutant T2 cell line were derived mostly from the invariant chain with the rest of the molecules being "empty." This defect suggests that an alternative pathway of loading peptides onto class II molecules is operating. A gp110 protein isolated from chemically induced BALB/c sarcomas has been shown to induce an antitumor cellular immune response in mice. The structure of its peptide fragments in association with class I molecules is being analyzed. The structures of both a synthetic 57-residue peptide comprising the double zinc finger of the human enhancer binding protein EPI and a 39-residue peptide containing the two zinc binding domains of the p7 nucleocapsid protein of HIV-1 have been determined by NMR.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

Major Findings:

I. T-Cell Antigen Recognition and Major Histocompatibility Complex (MHC) Antigens

1) Binding of endogenously processed peptides to Major Histocompatibility Complex (MHC) class I and class II molecules is central to the recognition of a wide variety of antigens by T-cells. The driving force behind the extraordinary allelic polymorphism exhibited by the MHC molecules is the necessity to bind to peptides derived from a wide spectrum of infectious agents. Because individuals express at most six Class I molecules out of the over 100 allelic forms so far identified, each MHC molecule is presumably capable of binding to a wide array of structurally distinct peptides. Previous studies have approached the issues of specificity and diversity in peptide binding through the use of synthetic peptides. Recent attention, however, has focused on the assessment of the properties and characteristics of naturally processed peptides bound to class I and class II MHC molecules. Our study has utilized the technique of electrospray ionization/triple quadruple mass spectrometry in order to achieve high resolution separation and direct identification of the peptides present in these complex mixtures. Furthermore, the mass spectrometric technique has provided the first quantitation of the amounts of individual peptides bound to MHC molecules, allowing the number of peptide-MHC complexes and the complexity of the entire peptide mixture to be estimated. Although such quantitation has been estimated indirectly by others, the mass spectrometric technique appears unique in its ability to provide this information in a direct way.

2) The data that we have so far obtained show the sequence of eight different natural peptides bound to the human MHC class I antigen, HLA-A2.1. Several of these sequences could be identified in normal cellular proteins, providing evidence that such proteins are processed and presented in the same way as viral proteins. Our data have also allowed the definition of a unique structural motif for peptides bound to HLA-A2.1. All peptides are 9 residues long and contain LEU/ILE at position 2. Another feature common to these peptides is a hydrophobic alklyl side chain at position 9. The length of the peptides is identical to those associated with a second HLA-B27 molecule (Jardetsky et al., Nature 353, 326 [1991]). However, the motif for peptides bound to HLA-A2.1 is entirely different from that of peptides bound to HLA-B27. This finding is highly relevant and emphasizes the specificity with which class I molecules bind peptides. The determination of other allele-specific motifs should provide an extremely valuable tool to predict and identify potential class I-restricted cytotoxic T cells (CTL) peptide epitopes that subsequently may be used as synthetic vaccines. Our study has also shown directly that all the peptides sequenced are present only in cells that express HLA-A2.1 and that synthetic peptides based on these sequences do bind to the HLA-A2.1 molecule. Thus, we have provided two separate criteria for the specific interaction of these peptides with the HLA-A2.1 class I molecule.

3) The antigenic peptides recognized by CTL's are derived from endogenous cytoplasmic proteins. These proteins are degraded by proteasomes and the peptides are then tranferred into the endoplasmic reticulum (ER) by specialized transport proteins. Once inside the ER, the peptides bind the class I MHC molecule and as a complex are carried to the cell's outer membrane. To better define this pathway, we have analyzed a mutant human cell-line, T2, defective in the machinery ordinarily needed for antigen presentation by class I MHC molecules. This cell line showed poor surface expression of transfected exogenous human class I HLA alleles; however, the endogenous HLA-A2.1 molecules

were expressed at reasonably high levels on the cell surface. With the use of electrospray ionization/tandem mass spectrometry technique we have found that in contrast to the large number of peptides associated with HLA-A2.1 on normal cells only seven dominant peptides were associated with HLA-A2.1 of the T2 mutant cells. These peptides all derived from "signal sequences" that are included in certain membrane bound and secretory proteins, and were also associated with HLA-A2.1 in normal cells. These results suggest that proteolysis of signal peptides in the ER is a second mechanism for processing and presentation of peptides with class I molecules. This new pathway may play a crucial role for some diseases in protecting us from pathogens that might have escaped the established antigen processing pathway.

4) MHC class II molecules bind peptides derived mainly from exogenous proteins that enter the cell by phagocytosis, endocytosis or internalization of part of the membrane. These exogenous proteins are processed in acidic vesicles and the resulting fragments associated with class II molecules are transported to the cell surface and recognized by helper T cells. As with class I, peptide binding induces a conformational change in a class II binding region, the $\alpha 1/\beta 1$ groove, as indicated by a distinct reactivity with monoclonal antibodies. Recently, sequences for twelve 13-17 residue peptides eluted from mouse class II I-A^b and I-E^b molecules have been reported. Each sequence was derived from cell membrane glycoproteins; each peptide varied in length and had different COOH-termini. We have obtained sequences from peptides eluted from another class II molecule, I-A^d by analysis with reverse phase HPLC and the combination of electrospray ionization/tandem mass spectrometry. Between 650 and 2,000 different peptides were associated with the class II molecule, I-A^d. Nine peptides were sequenced and all were derived from secretory or integral membrane proteins. They contained a six residue binding motif that was variably placed within the peptide chain. Because most of the peptides associated with class I MHC contain only nine residues, we determined if the longer class II peptides were of optimal length for high affinity binding to the I-A^d molecule. Truncated analogs of these peptides were synthesized and tested for their capacity to bind purified class II molecules. Our data indicate that the length of the naturally processed peptides is not the minimum required for strong binding. Unlike class I peptides, class II peptides can be shortened without losing appreciable binding activity. Crucial binding regions within the class II peptides are positioned variably within the observed sequences relative to their N- and C-Termini. Thus, for class II associated peptides, template-trimming to give unique termini is not necessary and an optimal occupancy of a polymorphic pocket may also not be required. Further sequence analysis should generate new information about the involvement of naturally processed peptides in specific infectious and autoimmune diseases.

5) Studies by others of human cell mutants have recently shown that the transporter genes, TAP1 and TAP2 mapped in the class II region of the MHC, are necessary for the formation, within the endoplasmic reticulum, of most class I/peptide complexes. The role of other genes present in the same class II region of the MHC important for class II-restricted antigen processing was investigated. Stable I-A^k and DR3 transfectants of a mutant cell line, T2, that have a large homozygous deletion within the MHC, including all the functional class II genes were obtained. These transfectants express parental levels of class II molecules at the cell surface, but fail to process and present epitopes derived from exogenous protein antigens. However, stimulation occurs if the appropriate peptides are provided. The DR3 molecules expressed in T2 do not have the epitope recognized by the specific monoclonal antibody 16.23 and dissociate more readily in the presence of SDS than the DR3 from a non-mutant cell line. These data indicate that the class II molecules,

although displayed in normal amounts by the mutant cell line, may be incapable of generating naturally processed class II peptide complexes. Interestingly, we have found a predominance of invariant chain peptides associated with the DR3 class II molecules from the mutant cell line. This predominance suggests two possibilities: 1) the production of most peptides that associate with DR3 molecules occurs in a cellular compartment different from the one in which the invariant chain derived peptides were produced; loading of most peptides requires fusion of the two compartments; 2) the production of most class II binding peptides from proteins other than the invariant chain is greatly impaired in the mutant cell line. Whatever the molecular explanation for the defect proves to be, it is clear that it probably results from the deletion of one or more genes encoded in the class II region of the HLA complex. These genes are located within a 230 kb DNA segment, but to date they are not known expressible genes.

6) Interferons (IFNs) are a family of specific polypeptides that affect a variety of biological responses. Interferon gamma (IFN-γ), a cytokine produced by activated T-lymphocytes, is a major regulator of specific immune responses. The first step of IFN's action is the binding to a specific cell surface receptor, leading to a signal transduced to the nucleus. The signal is followed by the induction of transcription of a number of genes, including class I and class II MHC genes. Promoters that respond to IFNs have an interferon consensus sequence (ICS) to which well-characterized proteins bind. Recently, a response element has been identified that contains a gamma interferon activation sequence. We have cloned a cDNA that codes for a binding protein with significant sequence similarity to several factors that bind the ICS. This factor is expressed predominantly in lymphoid tissues and is inducible preferentially by IFN-γ. This restricted expression suggests that this factor might have a specific role in mediating IFN response in cells of the immune system. Among the various factors which bind the same or very similar ICS sequence, a DNA binding domain of approximately 120 amino acids at the amino-terminal of the proteins has been identified. This domain consists of three repeats of approximately 50 amino acids and in each repeat there are three perfectly conserved tryptophans. To examine the role of the repeats in DNA binding, truncated constructs were tested for their ability to bind either ICS oligomers or mutated oligomers. Our data indicate that the repeats are essential for the DNA binding and may represent a characteristic property of this group of DNA-binding proteins. To investigate the role of our factors in regulating the transcription of MHC genes, a series of transient cotransfection experiments were performed. Constructs containing different portions of the murine or human MHC class I genes were tested in a series of CAT-assays in HeLa cells. Our results show that the IFN-γ inducible factor, in HeLa cells, is a trans-activating negative regulator. The CAT-activity was reduced by a factor of four in each instance, and, therefore, this binding factor might have a role in reducing the transcriptional activity of IFNs inducible genes. Several experiments are being performed in an attempt to unravel the role of this nuclear factor in the complexed machinery of IFNs mediated gene regulation.

7) One of the important events in T-cell development is intrathymic maturation. In the thymus T-cells first express their receptors and mature to immune competence. About 40% of both the double negative T-cell precursors ($CD4^-/CD8^-$) and their progeny ($CD4^+/CD8^+$) are cycling. The precursor cells do not express the T-cell receptor and the mechanisms regulating their proliferation must be different from that of the differentiated intrathymic or peripheral $CD4^+$ T-cells. We have demonstrated that an antigen on $CD4^-/CD8^-$ thymocytes when cross-linked by a specific monoclonal antibody triggers thymocytes proliferation. This antigen is dipeptidyl dipeptidase IV (CD26). The CD26 expression is developmentally regulated in both lymphoid and

hemapoietic cells. The responsiveness to stimulation by CD26 is present not only with CD4-/CD8- cells but also with bone marrow cells and more differentiated cells. This suggests the existence of a regulatory mechanism of potential target for pharmacological agents aimed at increasing granylocyte, macrophage and T-cell production in vivo.

II. Tumor specific transplantation antigens

Individually distinct tumor specific transplantation antigens (TSTA) are expressed in chemically-induced tumors. We have purified two TSTAs from the 3-methylcholanthrene-induced murine fibrosarcoma, Meth A. One TSTA consists of two closely-related proteins of 84 and 86 kDa. These proteins were identified as the murine equivalent of the 90 kDa heat shock proteins. The other TSTA previously referred to as p82, has been identified as the murine homolog of human ezrin. These antigens have been detected and purified by following an in vivo tumor rejection assay. The lack of appropriate probes for detecting these antigens in in vitro immunological assay has hampered a detailed molecular characterization. We have identified a third antigen, gp110, which appears to be antigenically and chemically distinct from all previously identified Meth A antigens. Initial efforts have been directed toward the evaluation of the immunogenicity of gp110 in in vitro-based immunological assay systems. Highly enriched $gp110^{+}$ Meth A fractions, were shown to induce anti-tumor CMC activity in mice immunized with Meth A and CMS4 sarcomas. Purified preparations of Meth A and CMS4 gp110 presumably free of any potentially bound antigenic proteins and/or peptides, have been shown to stimulate the proliferation of the anti-Meth A and anti-CMS4 CTL cell lines. Research is currently in progress to identify the antigenic peptides associated with class I MHC molecules on the surface of Meth A and CMS4 and relate them to the structure of peptides bound to class I MHC. These studies should define the molecular basis for the antigenic diversity associated with these tumors.

III. AIDS Research

Regulatory proteins that bind DNA are extremely important as control elements of transcription, replication and certain types of recombination in living organisms ranging from viruses to higher eukaryotes. We have been interested in analyzing the structure of two cellular transcription factors, EPI and Nf-KB, which bind to two sites of the HIV-1 enhancer, one of which regulates transcriptional inducibility of the 5'LTR in activated T-cells. The EP1 binding protein has two widely separated pairs of Cys2His2 zinc fingers, each of which is capable of recognizing identical DNA sequences with similar affinities. Recently, we have shown that a synthetic 57-residue double zinc finger peptide from EP1 exhibits the same binding specificity and relative affinity as that observed for the intact protein. We have now determined the three dimensional solution structure of this peptide by NMR spectroscopy. An interesting finding is that the structures of the individual zinc fingers are very similar to those of other classical Cys2His2 fingers; however, the orientation of the two fingers in the free uncomplexed state is different from that observed in Zif-268, another triple zinc finger, in its mode of contact with DNA. This implies that the mode of contact with DNA in EP1 may be distinct from that observed in the Zif-268/DNA complex. Structural studies on the double zinc finger EP1-DNA complex by NMR have been started to further investigate this issue.

The GAG gene of HIV encodes a polyprotein precursor product that plays a major role in virus genomic RNA encapsidation and replication. The RNA binding activities are mediated by the nucleo capsid (NC) domain of their precursor

product. Mature NC is a 71 amino acid basic protein which contains two Cys3His type zinc fingers. A 39-residue peptide containing the two zinc fingers has been prepared by solid-phase peptide synthesis and the solution structure characterized by circular dichroism and NMR. The peptide exhibits a random coil conformation in absence of zinc but appears to form an ordered structure in the presence of zinc. The solution structure of the zinc complex consists of a β-hairpin at the N-terminus followed by a loop which is tethered to the zinc atom by the histidine and last cysteine side chain. The two fingers are separated by a very flexible linker segment which contains four basic residues. A second peptide of 55 residues was synthesized and fluorescence studies were carried out. The synthetic peptides showed an increase in β-turn content upon addition of zinc. The tryptophan fluorescence quantum yield also increased, typical of a solvent-exposed freely-rotating chromophore. Further experiments are being carried out with the complete nucleocapsid protein of 71 amino acids overexpressed in Escherichia coli from an inducible phage T7 RNA polymerase promoter. A detailed analysis of its RNA-binding properties is underway in order to unravel the various stages important in viral replication.

Publications:

Giacomini P, Ciucci A, Nicotra MR, Nastruzzi C, Barbieri R, Appella E, Gambari R, Pozzi L, Natali PG. Tissue specific expression of the HLA-DRα gene in transgenic mice (HLA-DRα/transgenic mice), Immunogenetics 1991;34:385-391.

Joshua-Tor L, Appella E, Frolow F, Rabinovich D, Sussman JL. Refined 3-D structure of bulge containing DNA, J Mol Biol 1992;225:397-431.

Demotz S, Sette A, Sakaguchi K, Buchner R, Appella E, Grey HM. Self peptide requirement for class II MHC allorecognition, Proc Natl Acad Sci USA 1991;88:8730-8734.

Moore SK, Appella E, Villar JV, Kozak CA. Mapping of the mouse 86-kDA heat-shock protein expressed gene (Hsp86-1) on chromosomes 12 and related genes on chromosomes 3,4,9, and 11, Genomics 1991;10:1019-1029.

Ullrich SJ, Moore SK, Law LW, Vieira WD, Sakaguchi K, Appella E. The role of HSP90 in tumor specific immunity. In: Maresca B, Lindquist S, eds. Heat Shock. Berlin: Springer-Verlag, 1991;269-78.

Grohmann U, Puccetti P, Romani L, Binaglia L, Bianchi R, Ullrich SJ, Appella E, Fioretti MC. Immunogenic tumor variants induced by drug treatment of the L5178Y lymphoma: search for serologically defined antigens at the clonal level. Intl. J. Cancer (in press).

Henderson RA, Michel H, Sakaguchi K, Shabanowitz J, Appella E, Hunt DF, Englehard VH. HLA-A2.1-Associated peptides from a Mutant Cell Line: A second pathway of antigen presentation. Science 1992;235:1264-1266.

Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science 1992;235:1261-1263.

Marks MS, Levi B.-L., Segars JH, Driggers PH, Hirschfeld S, Nagata T, Appella E, Ozato K. H-2RIIBP expressed from a baculovirus vector binds to multiple hormone response elements. Molecular Endocrinology 1992;6:219-230.

Marks MS, Hallenbeck PL, Nagata T, Segars JH, Appella E, Nikodem VM, Ozato K. H-2RIIBP (RXRb) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thryroid hormone responsive gene. The EMBO Journal 1992;11:1419-1435.

Shlyakhtenko LS, Lyubchenko YL, Appella E, Harrington RE. CA runs increase DNA felxibility in the complex of lambda cro protein to the O_R3 site. Science (in press).

Bristol LA, Sakaguchi K, Appella E, Doyle D, Takacs L. Thymocyte co-stimulating antigen is CD26 (dipeptidyl-peptidase IV): co-stimulation of granulocyte, macrophage and T-lineage cell proliferation via CD26. J. Immunology (in press).

Omichinski JG, Clore GM, Sakaguchi K, Appella E, Gronenborn AM. Structural characterization of a 39-residue synthetic peptide containing the two zinc binding domains from the HIV-1 p7 nucleocapsid protein by CD and NMR spectroscopy. FEBS 1991;292:25-30.

Omichinski JG, Clore GM, Robien M, Sakaguchi K, Appella E, Gronenborn AM. High-resolution solution structure of the double Cys2His2 zinc finger form the human enhancer binding protein MBP-1. Biochemistry 1992;31:3907-3917.

Robien MA, Clore GM, Omichinski JG, Perham RN, Appella E, Sakaguchi K, Gronenborn AM. Three-dimensional solution structure of the E3-binding domain of the dihydrolipoamide succinyltransferase core from the 2-oxoglutarate dehydrogenase multienzyme complex of Eschericia coli. Biochemistry 1992;31:3463-3471.

Hunt DF, Michel H, Dickinson T, Shabanowitz J, Cox AL, Sakaguchi K, Appella E, Grey H, Sette A. Peptides presented to the Immune System by the Class II MHC molecule, $I\text{-}A^d$. Science (in press).

Olson D, Pöllänen J, Rønne E, Gunilla H-H, Wun T-C, Sakaguchi K, Appella E, Danø K, Blasi F. On the mechanism of internalization of the urokinase:PAI-1 complex. JBC (in press).

Flanagan JR, Becker KG, Ennist DL, Gleason SL, Driggers PH, Levi B-L, Appella E, Ozato K. A novel zinc-finger protein binds the upstream conserved region of Moloney Murine Leukemia virus. Molecular and Cellular Biology 1992;12:38-44.

Duthu A, Debuire B, Romano J, Erhart JC, Fiscella M, May E, Appella E, May P. p53 mutations in raji cells: Characterization and localization relative to other burkitt's lymphomas. Oncogene (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05597-03 LCBGY

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Biochemistry of Energy-Dependent (Intracellular) Protein Degradation

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

PI:	M.R. Maurizi	Special Expert	LCB, NCI
Other:	M.W.Thompson	IRTA Fellow	LCB, NCI
	S.K. Singh	Fogarty Fellow	LCB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

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

ATP-dependent proteases are responsible for a major portion of the degradation of intracellular proteins in eukaryotic and prokaryotic cells. The *E. coli* Clp protease is representative of a universal family of ATP-dependent proteases that are composed of a proteolytic core (ClpP) and several ATP-dependent regulatory subunits (ClpA-family members). Interactions between the regulatory subunits and the proteolytic subunit may affect the specificity and activity of the Clp proteases in vivo. Our research has focused on the biochemistry of E. coli Clp protease with the aims of (1) defining the specificity of the protease and the mechanism by which it selects targets in vivo, and (2) defining the functions of ATP in the selection and degradation of proteins. We have succeeded in clearly defining two roles for ATP. ATP acts as an allosteric effector for the assembly of the complex between the regulatory component, ClpA, and the proteolytic component, ClpP. Interaction between ClpA and ClpP in turn alters the active site of ClpP, indicated by the ability to cleave intermediate length peptides. Degradation of large proteins, however, requires ATP hydrolysis, indicating that ATP is required for a second step in degradation. The ATP-hydrolysis-dependent step probably involves alterations in the structure of large protein substrates (chaperone function) or changes in the interactions between the protein substrates and the enzyme (translocation function). Direct interaction between ClpA and proteins and peptides is indicated by the effects of these substrates on ATPase activity of ClpA in the absence of ClpP. Selectivity of proteolysis by Clp should involve interactions at both the active and the allosteric sites, since degradation of model peptide substrates indicates the the active site of ClpP has rather broad specificity. Site-directed mutagenesis of the ATPase site in domain 2 of ClpA indicates that hydrolysis at that site is not required for assembly of active Clp and cleavage of intermediate length peptides. Since mutants altered in domain 2 cannot degrade large proteins, that domain appears to be responsible for the processive steps in degradation by Clp.

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Major Findings:

(1) Earlier results indicated that ATP has two functions in activating Clp protease. ATP is required in an assembly reaction involving the association of ClpA subunits into a hexamer; the hexameric form of ClpA can interact with ClpP to promote proteolysis. Non-hydrolyzable analogs promote assembly of ClpA/ClpP but do not promote degradation of large proteins. We have now found intermediate length peptides that require the ATP-promoted ClpA/ClpP complex, but not ATP hydrolysis for degradation. Thus, nucleotide binding is sufficient to activate Clp for single peptide bond cleavage but cannot activate processive degradation of proteins. Moreover, with large proteins, not even limited degradation is observed, indicating that ATP hydrolysis is needed to affect the interaction between Clp and proteins in the initial phase of the reaction. We propose that ATP is used to drive either unfolding of proteins (chaperone function) or movement of proteins (translocation function) to position them in the active site for cleavage by ClpP. This function is needed for the initial interaction between Clp and large proteins and for the processive steps in completing the degradation. Studies are underway to mutagenize the ATP binding sites of ClpA both separately and together. The function of each ATP binding site for either self-assembly or activation of protein degradation will be investigated

(2) The sequence of Clp A reveals the presence of two ATP binding site consensus sequences, which are also found in the Clp A homologs of other organisms. The ATP binding sites occur in two separate regions that have little sequence homology to each other but are each well conserved in the different organisms. The existence of these two sequence regions suggests that Clp A is composed of two large structural domains, each with an ATP binding site as its primary functional feature. Our working model is that one domain is involved in the assembly reaction and the allosteric effect on the active site of ClpP and that the other domain is involved in interactions with the substrate to carry out the unfolding or translocation activities. Dr. S. K. Singh has made mutations in consensus amino acids in the ATP binding sites of both domains and shown that the sites in both domains are required for activation of large protein degradation. Both sites appear to have ATPase activity. Mutants lacking the ATP-binding site in domain 2 have been purified and shown to undergo self-assembly in the presence of ATP, suggesting that one function of domain 1 is to allow association of ClpA into hexamers. Domain 2 mutants can activate cleavage of intermediate length peptides but not large proteins, suggesting that domain 2 may be responsible for the folding or translocation function

(3) Dr. Mark Thompson has been investigating the specificity of Clp protease. He has been surveying families of related peptides as inhibitors of either protease or peptidase activity. Peptide inhibitors of Clp are of two classes: 1) those that inhibit both peptidase activity of ClpP and protease activity of the holoenzyme, and 2) those that inhibit protease activity but do not inhibit (or only weakly) peptidase activity. The former inhibitors bind at the active site thereby inhibiting cleavage of all substrates. In fact most of these inhibitors are alternative substrates for the enzyme. Peptides that do not bind at the active site but inhibit protease activity should define an allosteric binding site on Clp which would provide increased specificity in protein degradation, since potential substrates would have to be recognized at two sites by the protease to be cleaved. ClpA itself may have the allosteric site, because ATPase activity of ClpA is increased or decreased by a number proteins or peptides. Interaction between ClpA and ClpP may alter or increase the number of allosteric sites for proteins. Using a set of defined

pentapeptides, we have found that small peptides can fit into the active site of ClpP in different configurations to be cut at alternative sites. The rate of cleavage and the binding affinity of these peptides depends on the amino terminal amino acid, with neutral and hydrophobic residues being preferred.

(4) Clp P appears to undergo auto-processing *in vivo*, and information about the sequence or structural requirements for self-cleavage may help define the specificity of the protease. In collaboration with Dr. Susan Gottesman, we have made site-directed mutants of Clp P altered in amino acids around the processing site. Single amino acid substitutions at the P-1 position of the processing site as well as changes at the P1, P2, and P-2 positions have little or no effect on processing of ClpP in vivo. Several mutants, with substitutions at both P-1 and P-2 are processed more slowly than wild-type Clp P and also have lower proteolytic activity than the wild-type protease, suggesting that proper processing is required to allow Clp P to assume an active conformation. Since a purified unprocessed, mutant Clp P (Clp P-S11A) can adopt the native dodecameric structure and can interact with Clp A, the unprocessed or partial leader sequence in the processing-site mutants may interfere directly with the active site of the protease, as is the case with well-known zymogenic forms of other proteases. The 19 amino acid leader peptide has been synthesized in vitro and shown to be a highly specific substrate cleaved in an ATP-dependent reaction by Clp. Truncated forms of this peptide are also good substrates and have indicated that the substrate specificity lies within the five amino acids on either side of the processing site. Leader peptide is cleaved by Clp in the presence of non-hydrolyzable analogs of ATP, providing the most direct demonstration of the allosteric effect of nucleoside triphosphates in opening their active site of energy-dependent proteases. Recently it has been shown that E. coli has an N-end rule protein degradative system and that ClpA is required for the degradation of a set of fusion proteins defined by the amino terminal amino acid of the protein. We have initiated a collaboration with Dr. Alex Varshavsky to reconstruct the degradation reaction in vitro. Preliminary results indicate that ClpA/ClpP alone is not sufficient to degrade the sensitive fusion proteins, and an effort to identify other factors that may be required for recognition and degradation of these specific proteins is underway.

(5) In collaboration with Dr. Susan Gottesman, we have identified the open reading frame for a protein called ClpX, which has moderate homology to ClpA and may have an ATP binding consensus sequence. This protein has been purified by M. Zylicz (Warsaw, Poland) and shown to activate degradation of lambda O protein by ClpP in vitro. We have constructed an expression system that produces ClpX in quantity and are purifying the protein to study its role in Clp-dependent activities in vitro. These preliminary findings suggest that the Clp family is even larger and more complex than originally thought- E. coli possesses three related proteins, ClpA, ClpB, and ClpX. Possibly, interactions between ClpP and various proteins of the ClpA family may affect targeting and regulation of specific protein degradation in vivo.

(6) In collaboration with Dr. Lee Simon and his graduate student at the Waksman Institute, we have been been studying the interaction of a physiological inhibitor from bacteriophage T4 with Lon protease. This inhibitor, PinA, is a protein that forms a very tight complex with Lon protease (Kd ~ 1 nM) and inhibits both protease activity and basal level ATPase activity of Lon. Since the inhibitors does not inhibit peptidase activity of Lon it appears to interact at a site separate from the active site and may prove useful for identifying the allosteric site on Lon. The inhibitor also inhibits the substrate-activated ATPase of Lon and should help in studying the mechanism of coupling between ATP hydrolysis and peptide bond cleavage. PinA inhibits

Lon protease activity at a 1:1 molar ratio but requires higher amounts of inhibitor to block ATPase activity. This result suggests that only one allosteric site in the Lon tetramer need be occupied to affect proteolytic activity and suggests cooperativity between proteolytic active sites.

Publications:

Maurizi MR. Proteases and protein degradation in Escherichia coli. Experientia 1992;48:178-201.

Gottesman S, Maurizi, M. Regulation by proteolysis in prokaryotes and eukaryotes: energy-dependent proteases and their targets, Microbiological Reviews (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells

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

PI:	M.M. Gottesman	Chief, Laboratory of Cell Biology,	LCB, NCI
Co-PI:	I. Pastan	Chief, Laboratory of Molecular Biology	LMB, NCI

COOPERATING UNITS *(if any)*

A. Nienhuis	Chief, Clinical Hematology Branch	NHLBI, MH

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

B

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

Resistance to chemotherapy of human cancers has been studied by establishing *in vitro* and *in vivo* genetic systems which mimic development of drug resistance. A major mechanism of multidrug resistance in human cancer is expression of the *MDR*1 gene which we have cloned and sequenced. Expression of the *MDR*1 gene carried by retroviral vectors can be used to transduce cells to multidrug resistance and has been used to confer resistance to mouse bone marrow cells *in vitro* and *in vivo*. Mechanistic studies on P-glycoprotein suggest that it is involved in removing drug directly from the plasma membrane. To study mechanism, we have generated many mutants and initiated biochemical studies including the purification of P-glycoprotein and reconstruction of drug-dependent ATPase activity in artificial liposomes.

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Other Professional Personnel:

S. Altuvia	Special Volunteer	LCB, NCI
S. Goldenberg	Research Biologist	LCB, NCI
U. Germann	Visiting Fellow	LCB, NCI
I. Lelong	Visiting Fellow	LCB, NCI
P. Schoenlein	Staff Fellow	LCB, NCI
D.-W. Shen	Visiting Associate	LCB, NCI
I. Aksentijevich	Staff Fellow	LCB, NCI
C. Cardarelli	Research Biologist	LMB, NCI
J. Campain	Special Volunteer	LMB, NCI
G. Evans	Biotechnology Fellow	LMB, NCI
W. Stein	Special Volunteer	LMB, NCI
M. Siegsmund	Special Volunteer	LMB, NCI
J. Aram	Special Volunteer	LMB, NCI

Major Findings:

1. Transgenic mice which express the human *MDR*1 gene in their bone marrow have been used to evaluate various techniques for sensitizing multidrug resistant cells to chemotherapy. The assay system involves intraperitoneal injection of various agents, followed by a chemotherapeutic drug (usually daunorubicin, doxorubicin, or taxol) and a determination of peripheral white blood counts (WBC) 3 and 5 days after treatment. Resistant animals show no drop in WBC under these conditions, whereas animals sensitized to chemotherapy show drops in WBC. Under these assay conditions, verapamil is a potent chemosensitizing agent, and combinations of drugs such as quinine or quinidine with verapamil appear to be synergistic. Encapsulation of doxorubicin in liposomes improves the efficacy of this drug against MDR bone marrow. The monoclonal antibody MRK-16 can reverse multidrug resistance in vivo, but this effect is not synergistic with reversing effects of lower molecular weight agents such as verapamil. The immunotoxin MRK-16-PE (*Pseudomonas* enterotoxin) effectively kills MDR bone marrow in this model.

2. MDR bone marrow from transgenic mice can be transplanted into drug sensitive mice with complete transfer of the drug resistance phenotype. This result demonstrates that resistance resides in the bone marrow cells themselves, and suggests that the *MDR*1 gene may be an effective dominant selectable marker in gene therapy experiments. To test this hypothesis, isolated mouse bone marrow cells were infected with an *MDR*1 retrovirus and reintroduced into W/W-V mice which have hypoplastic bone marrow. Presence of the *MDR*1 gene in reconstituted animals could be detected for several months. Selection in vivo with taxol resulted in an increase in the number of peripheral neutrophils carrying the human *MDR*1 gene, indicating that these cells had a selective advantage in the presence of the cytotoxic drug taxol.

3. KB cell lines selected for resistance to the MDR drugs colchicine or vinblastine have amplified copies of the *MDR*1 gene. Pulsed field gel electrophoretic analysis of these MDR cells at various levels of selections demonstrates two different mechanisms of amplification, both of which proceed via extrachromosomal elements to form double minute chromosomes. In the first mechanism, found in colchicine selected cells, the earliest steps of amplification appear to involve small, cytogenetically non-detectable extrachromosomal elements of approximately 890 kb. As selection (and amplification) proceeds, this element doubles in size to approximately 1800 kb (which is cytogenetically detectable as a minute chromosome), and then doubles again to 3600 kb. Restriction digestion with Not 1, which cleaves only once in

the *MDR*1 amplicon, proves that this size increase results from two sequential dimerizations. In the second mechanism, seen in vinblastine-selected cells, the initial event is a formation of high molecular weight extrachromosomal element, which decreases in size and increases in copy number during selection in vinblastine.

4. P-glycoprotein has been purified to near homogeneity from plasma membrane vesicles prepared from MDR cell lines. The purification procedure involves solubilization in octyl glucoside, followed by column chromatography with DEAE and WGA. During this procedure, extraneous ATPase activities, including the Na/K ATPase found in plasma membrane, are separated from P-glycoprotein. The purified P-glycoprotein has some basal ATPase activity (approximately 1 umole/min/mg protein). When reconstituted into liposomes prepared from E. coli lipids, phosphatidyl serine, phosphatidyl choline and cholesterol, this basal activity can be stimulated several-fold by a variety of drugs known to be substrates for the P-glycoprotein efflux pump, including vinblastine and verapamil. Vanadate blocks both basal and stimulated ATPase activity.

5. Vesicles from MDR cells transport vinblastine in an ATP dependent manner. Addition of radioactive ATP or GTP results in phosphorylation of P-glycoprotein, predominantly on Ser residues. Non-radioactive GTP stimulates phosphorylation by ATP by an unknown mechanism. This phosphorylation of P-glycoprotein appears to be due to an exogenous kinase because purified P-glycoprotein does not autophosphorylate itself to any significant extent. Both protein kinase C and protein kinase A inhibitors have no effect on the exogenous phosphorylation of P-glycoprotein in this system, but staurosporine is a potent inhibitor, and vanadate stimulates phosphorylation. These results suggest the possibility that there is a novel kinase in plasma membrane vesicle preparations which phosphorylates P-glycoprotein.

6. We have continued a molecular genetic analysis of P-glycoprotein to define functional parts of the protein. Chimeras in which the first intracytoplasmic loop of P-glycoprotein is replaced by the homologous region from the *MDR*2 gene are non-functional. Replacement of 4 of 13 altered amino acids in these chimeras restores function. This result indicates that *MDR*2 does not differ that significantly from *MDR*1, and that it should be possible to use chimeras to define important functional regions of these two proteins. Substitution of the *MDR*2 amino terminal ATP site into *MDR*1 produces a fully functional transporter, indicating that this site in *MDR*2 is active in energy transduction.

7. We have inactivated one of two *mdr*1b genes in mouse adrenal Y-1 cells by homologous recombination. The *mdr*1b gene is normally expressed at high levels in these steroid-secreting cells, and we have speculated that it might be involved either in steroid secretion, or in protecting Y-1 membranes from the toxic effects of high concentrations of steroids. One *mdr*1b gene knock-out was obtained in which the second *mdr*1b allele was expressed at elevated levels compared to Y-1 parental cells. These results appear to provide a provocative association between *mdr*1b mRNA levels and steroid secretion in Y-1 cells.

8. VP-16 (etoposide) resistant human FEM-X melanoma cells have been isolated in several steps. They are cross-resistant to topoisomerase II active drugs, but not to other MDR drugs. However, isolated topoisomerase II has normal activity and is normally sensitive to VP-16 and other inhibitors. Resistance of topoisomerase II can be demonstrated in whole cells or in whole cell homogenates, but not in isolated nuclei. These results suggest that VP-16 resistance in these melanoma cells may be due to altered cellular bioavailability of VP-16, as could occur from metabolism, altered nuclear uptake, or compartmentalization.

9. High level cis-platinum resistant human KB cells and human hepatoma cells have been isolated. Cis-platinum resistance is associated with altered expression of many proteins as detected on 2D gels. Resistance from the human hepatoma cells can be transferred by DNA-mediated gene transfer into mouse Balb/c 3T3 cells where it appears to be linked to transfer of human repetitive Alu sequences, suggesting that this linkage might be used to isolate the human genes critical for development of cis-platinum resistance.

Publications:

Cenciarelli C, Currier SJ, Willingham MC, Thiebaut F, Germann UA, Rutherford AV, Gottesman MM, Barca S, Tombési M, Morrone S, Santoni A, Marianti M, Ramoni C, Dupuis ML, Cianfriglia M. Characterization by somatic cell genetics of a monoclonal antibody to the MDR1 gene product (P-glycoprotein): determination of P-glycoprotein expression in multi-drug resistant KB and CEM cell variants. Int J Cancer 1991;47:533-543.

Pastan I, Willingham MC, Gottesman, M. M. Molecular manipulations of the multidrug transporter: a new role for transgenic mice. FASEB J 1991;5:2523-2528.

Mickisch GH, Pastan I, Gottesman MM. Multidrug resistant transgenic mice as a novel pharmacologic tool. BioEssays 1991;13:381-387.

Gottesman MM Pastan I. The multidrug resistance (MDR1) gene as a selectable marker in gene therapy. In Cohen-Haguenauer O, Boiron M, eds. Human Gene Transfer. Vol 219. Colloque INSERM/John Libbey Eurotext, Ltd., 1991;185-191.

Pearson JW, Fogler WE, Volker K, Usui N, Goldenberg SK, Gruys E, Riggs CW, Komschlies K, Wiltrout RH, Tsuruo T, Pastan I, Gottesman MM, Longo DL. In vivo administration of MRK-16 monoclonal antibody reverses drug resistance in a human colon cancer xenograft expressing the MDR1 cDNA. [Report] J Natl Cancer Inst 1991;83:1386-1391.

Reddy PG, Graham GM, Datta S, Guarini L, Moulton TA, Jiang H, Gottesman MM, Ferrone S, Fisher P. Effect of recombinant fibroblast interferon and recombinant immune interferon on growth and the antigenic phenotype of multidrug-resistant human glioblastoma multiforme cells. J Natl Cancer Inst 1991;83:1307-1315.

Weaver JL, Pine PS, Aszalos A, Schoenlein PV, Currier SJ, Padmanabhan R, Gottesman MM. Laser scanning and confocal microscopy of daunorubicin, doxorubicin, and rhodamine 123 in multidrug-resistant cells. Exp Cell Res 1991;196:323-329.

Mickisch GH, Licht T, Merlino GT, Gottesman MM, Pastan I. Chemotherapy and chemosensitization of transgenic mice which express the human multidrug resistance gene in bone marrow: efficacy, potency and toxicity. Cancer Res 1991;51:5417-5424.

Gottesman MM, Schoenlein P, Currier S, Bruggemann E, Pastan I. Biochemical basis for multidrug resistance in cancer. In: Pretlow G II, Pretlow P, eds. Biochemical and Molecular Aspects of Selected Cancers. Vol. 1. San Diego, Academic Press, Inc, 1991;339-371.

Lelong IH, Guzikowski AP, Haughland RP, Pastan I, Gottesman MM, Willingham M C. A fluorescent verapamil derivative for monitoring activity of the multidrug transporter. Mol Pharmacol 1991;40:490-494.

Higgins CF, Gottesman MM. Is the multidrug transporter a flippase? Trends Pharmacol Sci 1992;17:18-21.

Chin K-V, Ueda K, Pastan I, Gottesman MM. Modulation of activity of the promoter of the human *MDR*1 gene by Ras and p53. Science 1992;255:459-462.

Mickisch GH, Aksentijevich I, Schoenlein PV, Goldstein LJ, Galski H, Stahle C, Sachs DH, Pastan I, Gottesman MM. Transplantation of bone marrow cells from transgenic mice expressing the human *MDR*1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice. Blood 1992;79:1087-1093.

Gottesman MM, Horio M, Lelong I, Handler J, Raviv Y, Galski H, Mickisch G, Merlino G, Willingham MC, Pastan I. Function of the multidrug transporter. In: Tsuruo, T, Ogawa M, eds. Drug Resistance as a Biochemical Target in Cancer Chemotherapy, Vol. 13, Chapter 3. Bristol-Myers Squibb Symposia Series, San Diego, Academic Press, Inc, 1992;45-62.

Gottesman MM, Pastan I. The multidrug transporter as a novel target in cancer chemotherapy. The Origins of Human Cancer: A Comprehensive Review. Cold Spring Harbor Symposium, September 4-10, 1990, New York: Cold Spring Harbor Laboratory 1991;845-853.

Schoenlein PV, Shen D-w, Barrett JT, Pastan I, Gottesman MM. Double minute chromosomes carrying the human MDR1 and MDR2 genes are generated from the dimerization of submicroscopic circular DNAs in colchicine-selected KB carcinoma cells. Mol Biol Cell 1992;3:507-520.

Mickisch GH, Rahman A, Pastan I, Gottesman MM. Increased effectiveness of liposome-encapsulated doxorubicin in multidrug-resistant transgenic mice compared with free doxorubicin. J Natl Canc Inst 1992;84:804-805.

Mickisch GH, Merlino GT, Alken PM, Gottesman MM, Pastan I. New potent verapamil derivatives that reverse multidrug resistance in human renal carcinoma cells and in transgenic mice expressing the human MDR1 gene. J Urol (in press).

Gottesman MM, Roninson IB, Pastan I. A molecular basis for multidrug-resistance in human cancer cells. In: Kessel D, ed. Resistance to Antineoplastic Drugs, CRC Press, Boca Raton, FL (in press).

Handler JS, Horio M, Willingham M, Pastan I, Gottesman MM. Polar localization of the multidrug resistance transporter in epithelia results in transepithelial transport of substrates. Elsevier Science Publishers, Amsterdam, The Netherlands (in press).

Goldstein LJ, Gottesman MM, Pastan I. Expression of the *MDR*1 gene in human cancers. Ozols RF, ed. Molecular and Clinical Advances in Anticancer Drug Resistance. Boston, Kluwer Academic Publishers (in press).

Padmanabhan R, Howard T, Gottesman MM, Howard BH. Use of Magnetic affinity cell sorting to isolate transiently transfected cells, multidrug resistant cells, somatic cell hybrids, and virally infected cells. Methods Enzymol (in press).

Kane S, Gottesman MM. Use of the multidrug resistance gene in mammalian expression vectors. Methods Enzymol (in press).

Lelong I, Padmanabhan R, Lovelace K, Pastan I, Gottesman MM. ATP and GTP as alternative energy sources for vinblastine transport by P-glycoprotein 170 in KB-V1 plasma membrane vesicles. FEBS Letters (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05599-02 LBBGY

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Tumor Suppressor Protein, p53

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

S.J. Ullrich	Prinicple Investigator	LCB, DCBDC, NCI
E. Appella	Medical Officer	LCB, DCBDC, NCI
M. Fiscella	Visiting Fellow	LCB, DCBDC, NCI
K. Sakaguchi	Visiting Associate	LCB, DCBDC, NCI
N. Zambrano	Visiting Scientist	LCB, DCBDC, NCI

COOPERATING UNITS *(if any)*

W.E. Mercer	Jefferson Cancer Institute, Philadelphia, PA
C.W. Anderson	Brookhaven National Laboratory, Upton, NY

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Chemistry

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	5	0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The structure and function of human wt p53 have been investigated in order to understand the anti-proliferation effect of wt p53 on cell growth. Analysis of the effect of wt p53 on cell-cycle and growth regulated gene expression revealed that expression of wt p53 did not affect expression of immediate early genes such as fos, myc and jun but did down-regulate transcription of several late G1 genes, b-myb, PCNA and DNA polymerase α. These results, together with our observation that p53 arrests cells in G1 prior to the restriction point, indicate that wt p53 acts to arrest cells at a specific point (mid to late G1 phase) in the cell cycle. Biochemical analysis of p53 found that growth arrest mediated by wt p53 correlated with a unique conformational and phosphorylation state of wt p53 compared to mutant p53. The increased phosphorylation was in the N-terminus of the protein and preliminary evidence suggests that it involves ser 15, a site phosphorylated by the DNA-activated protein kinase, as well as one or two additional serine residues. Site directed mutagenesis of this residue suggested that ser 15 is involved in regulation of p53 turnover.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864 939

Major findings:

1) Wt p53 is a nuclear phosphoprotein which behaves as an antioncogene. Missense mutations in p53 gene are associated with loss of p53 anti-oncogenic properties and gain of a positive transforming or oncogenic effect. Transfection experiments of mutant p53 and ras indicated that mutant p53 behaves as a dominant negative oncogene with respect to endogenous wt p53. This ability of mutant p53 to inhibit the function of wt p53 appears to be through the formation of ologomeric complexes with wt p53. Using mutant- and wt-specific p53 monoclonal antibodies, Pab 240 and 1620, respectively, it was shown that the cocomplex of mutant and wt p53 exists solely in the mutant conformation presumable via cooperative interaction between the mutant and wt proteins. Further, it has been proposed that wt p53 may exist in a mutant-like or positive proliferative conformation associated with cell growth, whereas upon growth arrest p53 exists in the growth-arresting conformation. The exact mechanism(s) whereby p53 exerts an anti-proliferative effect are not known but recent evidence suggests that p53 may act as a transcription factor.

2) Previously, our laboratory, in collaboration with Dr. W. E. Mercer, has shown that human wt p53 exerts an antiproliferative effect when expressed in a glioblastoma cell line stably transfected with an inducible wt p53 gene, despite the presence of mutant p53 in these cells; the cells were found not to proceed into S phase. We now have shown that the cells are arrested in G1 phase prior to or at the restriction point of the cell cycle. In order to determine the molecular mechanism of wt p53 action we have analyzed the effect of wt p53 on the expression of several cell cycle-regulated genes, including the immediate early genes c-fos, c-jun, myc; and late G1 phase genes PCNA, DNA polymerase α and b-myb. Wt p53 was found only to inhibit the expression of a subset of G1 gene transcripts: b-myb, PCNA and DNA polymerase α. Together with the cell cycle analysis, these data indicate that the p53 does not act on immediate early gene transcription but at a later point in the cell cycle. It is not known if p53 acts directly or indirectly to inhibit expression of PCNA, DNA polymerase α and b-myb. Nuclear run on experiments indicate p53 affects transcription of these genes. Experiments are underway to examine the promoter region of these genes to determine the negative regulatory regions and if wt p53 interacts directly with these regions.

3) Study of wt p53 protein has been hindered by its low level of expression and high turnover rate; however, the inducible expression of high levels of wt p53 in the GM47 cell line has allowed us to study the biochemical properties of wt p53 protein. Using monoclonal antibodies, two major pools of p53 were detected 2h after the induction of wt p53 expression; one pool contained wt-p53 and mutant p53 in a mutant conformation and the other pool contained free wt-p53. As the G1-arrest developed, the amount of wt-p53 associated with the mutant pool decreased, so that by 12h, the major pools consisted of free wt p53 and the other only contained mutant p53. The wt p53 pool was distinguished from the mutant p53 pool by differential reactivity with various monoclonal antibodies; the wt p53 pool was $1620^{+}/421^{-}/1801^{+}/240^{-}$, whereas mutant p53 was $1620^{-}/421^{+}/1801^{+}/240^{+}$, implying they exist in different conformations. Moreover, 2D gel analysis indicated that the wt p53 pool can be distinguished by a greater degree of phosphorylation compared to mutant p53. Thus, the ability of wt p53 to exert its anti-proliferative effect is characterized by increased phosphorylation and loss of the Pab 421 epitope whereas mutant p53 apparently cannot undergo these postranslational modifications found in the wt protein.

4) Phosphopeptide mapping of wt p53 and mutant p53 indicated that wt p53 was phosphorylated to a greater degree at the N-terminus of the protein between amino acids 1-24, and to a lesser degree at the Ser 392 C-terminal site. No significant difference was found at the $p34^{cdc2}$ protein kinase site. Interestingly, the first 42 residues of p53 encode a transactivating region. Furthermore, located in this N-terminal region of p53 are five potential phosphorylation sites at Ser 6, 9, 15, 20 and 37. We have found in collaboration with Dr. C.W. Anderson's laboratory that Ser 15 and 37 are phosphorylated *in vitro* by the DNA-activated protein kinase (DNA PK). Other investigations have shown that Ser 9 is phosphorylated *in vitro* by casein kinase I like activity. Site directed mutagenesis of the Ser 15 and 37 sites of p53 either individually or together have been made and these plasmids have been stably transfected into T98G cells under the inducible promoter, MMTV. Cells expressing Ser 37 mutants behave like wt p53 in ability to induce growth arrest and in their protein steady state levels. Preliminary results with the ser 15 mutant indicate that it has a reduced synthesis and decreased steady state level despite a high level of expression of the transcript. Growth arrest assays indicate it has a minor reduction in activity compared to wt p53. Furthermore, the Ser 15 mutant has a much longer half-life compared to either wt p53 or the endogenous Ile 237 mutant p53 expressed in T98G cells. The importances of these sites in terms of p53 function are being explored.

Publications:

Mercer WE, Shields MT, Lin D, Appella E, Ullrich SJ. Growth suppression induced by wild type p53 protein is accompanied by selective down regulation of proliferating cell nuclear antigen (PCNA) expression. Proc. Natl Acad Sci USA, 1991; 58: 1958-62.

Lehman TA, Bennett WP, Metcalf RA, Welsh JA, Ecker J, Modali RV, Ullrich S, Romano JW, Appella E, Testa JR, Gerwin BI, Harris CC. p53 mutations, *ras* mutations, and p53-heat shock 70 protein complexes in human lung carcinoma cell lines. Cancer Res. 1991; 51: 4090-96.

Ullrich SJ, Mercer WE, Appella E. Human wild-type p53 adopts a unique conformational and phosphorylation state in vivo during growth arrest of glioblastoma cells. Oncogene 1992, in press.

Mercer WE, Ullrich SJ, Shields MT, Lin D, Alde H. Cell cycle effects of microinjected antisense oligonucleotides to $p34^{cdc2}$ kinase. Anals NY Acad Sci 1992, in press.

Lin D, Shields MT, Ullrich SJ, Appella E, Mercer WE. Growth arrest induced by wild type p53 protein blocks cells prior to or near the restriction point in late G1-phase. Proc Natl Acad Sci USA 1992, in press.

Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E, Anderson CW. The human DNA-activated protein kinase phosphorylates serine 15 and 37 in the aminoterminal transactivation domain of human p53. Mol Cell Biol (in press).

Ullrich SJ, Anderson CW, Mercer WE, Appella E. The p53 tumor suppressor protein - A modulator of cell proliferation. J Biol Chem (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08705-16 LCBGY

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Genetic and Biochemical Analysis of Cell Behavior

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

PI:	M.M. Gottesman	Chief, Laboratory of Cell Biology,	LCB, NCI
Other:	S. Goldenberg	Research Biologist	LCB, NCI
	R. Fleischmann	Senior Staff Fellow	LCB, NCI
	D. Ray	Research Biologist	LCB, NCI
	M. Gosse	PRAT Fellow, Sr. Staff Fellow	LCB, NCI

COOPERATING UNITS *(if any)*

G. Merlino	Visiting Expert	LMB, NCI
C. Jhappan	Associate	LMB, NCI

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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

☐ (a1) Minors B

☐ (a2) Interviews

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

The Chinese hamster ovary (CHO) fibroblast is an excellent model system for the study of the genetics and biochemistry of some aspects of the behavior of cultured cells. We are using this system to analyze the manner in which cyclic AMP regulates cell growth and gene expression in mammalian cells. The mechanism of cAMP action of CHO cells has been studied by generating cell lines resistant to growth inhibition by cAMP. One way to develop resistance to cAMP is through mutations in cAMP-dependent protein kinases which result in either altered regulatory (RI) or catalytic (C) subunits. Defects in cAMP-dependent protein kinase block cAMP-stimulated transcription and reduce amounts of mRNA for the multidrug transporter in CHO cells and in adrenal Y-1 cells. We have constructed several mutant forms of the CHO RI subunit and a mutant C subunit and expressed these in *E.coli*. Bacterially expressed mutant RI's affecting sites for recognition of RI by C and for binding of cAMP to RI have defects in associating with the C subunit and in cAMP-stimulated dissociation of RI and C subunits. An improved system for the inactivation of cAMP dependent protein kinase in mammalian cells has been developed. PCR-directed mutagenesis of the CHO RI cDNA cloned into a mammalian expression system is done to create a RI mutant library which can be electroporated into CHO cells followed by selection of dominant negative mutants. This vector system promises to be useful for analysis of cAMP effects in mammalian cells.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Major Findings:

1. We have cloned active CHO RI and C subunits from cAMP dependent protein kinase and expressed them at high levels in E. coli using the Studier expression system. RI is both soluble and active in binding; Cα, a C subunit isozyme, is partially soluble and active as a kinase. Both soluble subunits can be reconstituted to form active holoenzyme in vitro and both subunits have been used as antigens to generate specific antisera in rabbits. Six mutant RI proteins were generated by in vitro site-directed mutagenesis and have been analyzed for their ability to inhibit C subunit and by the ability of cAMP to bind to RI and relieve its inhibition of C. Two mutations, GE201 and YF371 (glycine for glutamine at amino acid position 261 and tyrosine for phenylalanine at amino acid position 371) are identical to mutations previously described in mouse and bovine RI proteins. Four novel mutations are: WR261, YG376, VA89, and GStop 200. All six mutant RI's as well as wild type RI were partially purified from E. coli and were studied for cAMP binding and cAMP mediated activation of mutant RI/Cα holoenzyme. The cAMP binding site mutants WR261, GE201, and YF370 all demonstrated a 5-fold decrease in affinity for cAMP as well as a 3-fold decrease in the ability of cAMP to activate the mutant RI/Cα holoenzyme. VG376, near a cAMP binding site mutant. Thus, this mutation was had no phenotype a conservative change which did not alter the functioning cAMP interaction with RI. The mutation VA89, a mutation in the putative R-C interaction site, weakened the interaction with Cα as indicated by a 3-fold lower EC_{50} for mutant holoenzyme activation by cAMP. The truncation mutant, GStop200, which lacks both cAMP binding sites, did not bind cAMP but did interact with and inhibit Cα or subunit activity. It appears that this novel deletion mutant may be an excellent candidate for a dominant negative mutant which could inactivate C in mammalian cells.

2. We have used a second novel approach to isolate dominant negative mutants of RI for use as moveable genetic elements for the inactivation of cAMP dependent protein kinase in mammalian cells. In this approach, a full-length CHO RI subunit is mutagenized using PCR under conditions in which one deoxynucleotide is limiting. A library of these mutated RI's was then cloned downstream from a powerful CMV promoter in the vector pRc/CMV which also carries a G418 resistance element under control of the SV40 promoter. This library was electroporated into CHO cells, which were subsequently selected with G418. G418 resistant clones were further selected in cholera toxin and several cAMP resistant colonies were obtained. The RI in these colonies was rescued by PCR, and sequence analysis indicated that at least one of these RI's has multiple mutations affecting cAMP binding site B in RI. Re-introduction of this mutant RI into CHO cells reliably produces a cAMP resistant phenotype, suggesting that this new mutant RI will be a useful new tool for analysis of cAMP effects in mammalian cells.

3. Using existing cAMP resistant mutants with alterations in cAMP dependent protein kinase, we have analyzed the requirement of this kinase in transcription stimulated by cAMP. Our model system involves transfection of CAT vectors driven by a 3.0 kb segment of the rat tyrosine amino transferase (TAT) promoter. This promoter segment does not contain a classic cAMP response element (CRE), but is stimulated several-fold by cAMP in CHO cells. This stimulation is completely blocked in various kinase mutants, indicating that non-classical cAMP transcriptional responses appear to be mediated via phosphorylation, as do responses mediated through CRE's.

4. We have continued to analyze the involvement of cAMP dependent protein kinase in regulating mRNA levels for the multidrug resistance (*mdr*) gene. Kinase deficient mutants of mouse adrenal Y1 cells have dramatically reduced

levels of *mdr*1b mRNA, and expression of C subunit activity in these mutants increases the amount of *mdr*1b mRNA. No alterations in transcription can be detected in these cells, suggesting the possibility that cAMP dependent protein kinase regulates the stability of mdr1b mRNA.

Publications:

Kessin RH, Fleischmann RD, Gottesman MM, Jastorff B, Van Lookeren Campagne MM. Use of the yeast low Km phosphodiesterase gene to control cAMP levels in mammalian cells, 1989 Japenese Meeting on Phosphodiesterases 1991; In: Strada SJ, Hidaka H, eds. Advances in Second Messenger and Phosphoprotein Research, Vol. 25. New York, Raven Press, Ltd, 1992;13-27.

Howard P, Day KH, Kim KE, Richardson J, Thomas J, Abraham I, Fleischmann R D, Gottesman MM, Maurer RA. Decreased catalytic subunit mRNA levels and altered catalytic subunit mRNA structure in a cAMP-resistant Chinese hamster ovary cell line. J Biol Chem 1991;266:10189-10195.

Fleischmann RD, Jeng C, Gottesman MM. Ablation of stimulation of a cAMP-responsive promoter in CHO cell lines defective in their cAMP-dependent protein kinase system. Som Cell Mol Genet, 1992;18:103-111.

Chin KV, Chauhan SS, Abraham I, Sampson KE, Krolczyk Aj, Wong M, Schimmer B, Pastan I, Gottesman MM. Reduced mRNA Levels for the Multidrug-Resistance Genes in cAMP-Dependent Protein Kinase Mutant Cell Lines. J Cell Physiol (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08715-14 LCBGY

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Synthesis and Function of a Transformation-Dependent Secreted Lysosomal Protease

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

PI:	M.M. Gottesman	Chief, Laboratory of Cell Biology	LCB, NCI
Other:	S. Goldenberg	Research Biologist	LCB, NCI
	D. Ray	Research Biologist	LCB, NCI
	S. Chauhan	Visiting Fellow	LCB, NCI
	J. Reed	Medical Staff Fellow	LCB, NCI
	V. Hearing	Research Biologist	
	K. Urabe	Visiting Fellow	LCB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Procathepsin L (also known as MEP, for major excreted protein) is a lysosomal protease which is synthesized and secreted in large amounts by malignantly transformed mouse and human cells, as well as by normal cells of the kidney and liver. Cathepsin L is a cysteine acid protease with a broad substrate specificity which includes many extracellular matrix substrates. To understand the mode of regulation of cathepsin L, we have isolated both the mouse and human genes and studied their upstream, promoter regions. The mouse promoter has elements both 5' and 3' to the start site of transcription which affect regulation by the tumor promoter, TPA. The human gene, which maps to chromosome 9, appears to have two distinct promoters which determine expression of two transcripts in most human tissues. A deletion analysis of a full-length human procathepsin L cDNA expressed in cultured mouse cells has revealed many features of the protein involved in subcellular localization and function. Sequences in the amino terminal section of the protein appear to be involved in protein folding and their deletion results in trapping of procathepsin L in the endoplasmic reticulum. Amino acid residues at the carboxy terminal end of cathepsin L affect its ability to be secreted.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Major Findings:

1. We have used two approaches to isolate the human cathepsin L gene. In the first, a human cathepsin L cDNA probe was used to screen a human cosmid library prepared from KB (HeLa) cell DNA. Several cosmids were isolated by this approach, but none of them showed exon sequences which corresponded exactly to sequences in the cDNA. Because of the presence of stop codons within these presumptive coding sequences, we assume that these cosmid isolates correspond to cathepsin L pseudogene(s) within the human genome. In a second approach, we used genomic PCR to isolate segments of the human cathepsin L gene which corresponded exactly to our cDNA probes and were able to reconstruct the entire coding region of the functional human cathepsin L gene, which maps to chromosome 9.

2. Our original human cathepsin L cDNA differed in its 5' sequence from a cathepsin L cDNA published by another laboratory. The divergence in sequence began just upstream from the codon specifying the initiating methionine. Our analysis of the upstream region of our genomic cathepsin L clones has resulted in an explanation for this discrepancy: one of the mRNAs is transcribed from a promoter which is contained within the first intron of a transcription unit which is driven by a promoter further upstream. Thus, the human cathepsin L gene has two promoters, which may be differentially regulated.

3. We have begun to explore the possibility that cathepsin L, which is secreted in transformed mouse cells, and cathepsin B, a cysteine protease with similar substrate specificity, may be involved in invasiveness and metastasis of cancers. In collaboration with the laboratory of Dr. Vincent Hearing, we have tested several lines of mouse B16 melanoma cells isolated on the basis of their differing metastatic potential. With respect to levels of cathepsin B or L mRNA, their appears to be no correlation with metastatic potential.

4. To obtain more definitive information about the possible requirement of cysteine proteases for metastasis, we have begun a project to inactivate these genes in mouse melanoma cells by homologous recombination. To make the cloning of genomic segments of these genes easier, and to facilitate the isolation of homologous recombinants, we have designed a novel "gene knockout" vector with the following features: (1) multiple unique restriction sites around a cassette which confers G418 resistance for positive selection; (2) HSV-TK cassettes at either end for negative selection using ganciclovir; (3) Unique, rare (SfiI) restriction sites on either side of the HSV-TK cassettes for linearization of the "knock-out" DNA. Several kb of both cathepsin L and cathepsin B genomic DNA have been cloned in this vector.

Publications:

1. Chauhan S, Gottesman MM. Construction of a new universal vector for insertional mutagenesis by homologous recombination. Gene (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09100-7 LCBGY

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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
Other:	K. Urabe	Visiting Fellow	LCB, NCI
	P. Aroca	Guest Researcher	LCB, NCI
	L. Law	Scientist Emeritus	LG/NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
3.5	2.5	1

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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, with respect to their outgrowth and metastasis. Our studies have identified, isolated and characterized three different melanogenic enzymes that interact to regulate the quality and quantity of pigment produced within melanocytes. These proteins share significant sequence homology and belong to a new family of tyrosinase-related genes that are specifically expressed by mammalian melanocytes. In combination with a melanogenic inhibitor currently being studied which affects those catalytic activities, mammalian melanogenesis is strictly regulated. Our studies have also continued the characterization of melanoma-specific antigens abnormally expressed on transformed melanocytes. At least two of those antigens are related to normal melanocyte constituents which are aberrantly expressed or synthesized by the transformed cells; those antigens are recognized by the tumor-bearing host as foreign. Monoclonal antibodies to those antigens have now been raised which cross-react with human melanoma cells; these highly specific reagents are proving useful in immunodiagnostic tests, and in experimental anti-metastatic assays in murine models.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

Major Findings:

1. Melanogenesis: We have continued our studies into regulatory factors important to the expression of melanocyte function, i.e. melanogenesis. Five different pigment related genes have now been identified by various laboratories using alternate strategies to clone the gene for tyrosinase; those genes encode homologous proteins with highly conserved predicted structural and functional properties. We have synthesized peptides corresponding to divergent regions at the carboxyl and amino termini of those predicted protein sequences, and have raised specific antibodies against them. Those antibodies have been shown to recognize the expected proteins synthesized *in vivo* and *in vitro* and have been used for their purification and characterization. We have demonstrated that in spite of their overall similarity at the structural level, each of those proteins has a unique enzymatic function. Tyrosinase, which maps to the *albino* locus in mice, has three separate catalytic activities - the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA), the oxidation of DOPA to DOPAquinone, and the oxidation of dihydroxyindole (DHI) to indole-quinone. TRP2 (DOPAchrome tautomerase), which maps to the *slaty* locus in mice, has yet another distinct catalytic function, the isomerization of DOPAchrome to dihydroxyindole-2-carboxylic acid (DHICA). We have now shown that melanins synthesized *in vitro* and *in vivo* are rich in carboxylated intermediates, although the significance of this to the structure and function of the melanin is currently unknown, but is being actively investigated by our group. Tyrosinase and DOPAchrome tautomerase have independent activities and there is no residual activity in either protein towards the other's substrates. Pmel17 (TRP3, stablin) maps to the *silver* locus in mice, and encodes a catalytic activity that functions even more distally in the melanogenic pathway, regulating the further metabolism of DHI and DHICA. TRP1, which maps to the *brown* locus in mice, has catalytic functions identical with tyrosinase, but with a specific catalytic activity only 5 to 10% that of tyrosinase. However, the major function of TRP1 appears to be through its interaction with the other proteins, where it dramatically increases their stability. This suggests that *in vivo*, the association of these melanogenic proteins within the melanosomal matrix may prove important to their activation and stability, and thus their catalytic function. Another important regulator of mammalian melanogenesis is a low molecular weight inhibitor that inhibits the activities of these melanogenic enzymes, with the end result of a dramatic decrease in melanin pigmentation. This inhibitor has been isolated from unpigmented murine melanomas *in vivo*, which have normal levels of melanogenic enzymes. The inhibitor has also been purified from amelanotic human melanoma sources, and is currently being chemically defined. We are concurrently collaborating on studies aimed at defining the functional motifs of tyrosinase using cloned spontaneous tyrosinase gene mutations where more than 30 distinct spontaneous mutations have thus far been determined which result in the human albino phenotype. Those studies are also being directed at eventually determining which of the other cloned pigment related loci may be involved in different types of hypo- and hypermelanotic abnormalities.

2. Melanoma Biology and Immunology: Our studies on the characterization of the antigens responsible for the generation of melanoma specific transplantation responses has continued to emphasize the importance of the B700 antigen in those responses. We have shown that melanoma-bearing mice produce specific complement-dependent cytotoxic antibodies which have the same

specificity elicited by immunization protocols with various melanoma tumors; those antibodies are specifically directed against the B700 antigen on the melanoma cell surface. A vaccine derived from formalinized extracellular melanoma antigens has been shown to be effective in eliciting melanoma-specific tumor rejection; antibodies in those immunized animals are predominantly directed against the B700 antigen. We have successfully developed murine monoclonal antibodies specific for B700 which also specifically cross-react with human melanomas (36/37, including amelanotic and spindle cell melanomas), and some pigmented nevi (4/7), but do not cross-react with nonmelanomatous tumors (26/28, 1 breast carcinoma and 1 spindle cell carcinomas were weakly positive) or with normal skin (0/2). Those monoclonal antibodies have also proven useful in treating established pulmonary metastases in mice; 50 to 75% decreases in the incidence of metastatic outgrowth of melanoma cells can be routinely elicited by treatment of animals with the purified, unconjugated, monoclonal antibodies 5 to 8 days after challenge with tumor cells. This effect seems to result from the increased activity of natural killer cells through enhanced recognition of the antibody coated tumor cells, although cytotoxic T cells and other effector mechanisms may also participate in these responses. These antimetastatic effects can also be elicited by treatment with other monoclonal antibodies which recognize yet another surface antigen of murine melanoma cells termed 9B6; these latter antibodies however have no cross-reactivity with human melanoma cells, and thus their potential usefulness in immunodiagnosis or immunotherapy of human melanomas is not as significant as those which recognize B700. In related studies which have attempted to clarify the mechanism by which tumor antigens are generated, we have further demonstrated the relationship of melanoma associated antigens with aberrantly expressed normal cellular surface membrane constituents. In the case of B700, the relationship is to the serum albumin family (which includes other surface proteins such as _-fetoprotein and vitamin D binding protein), and in the case of B50, the relationship is to a family which includes calcium binding proteins and the Ro/SS-A antigen of human systemic lupus erythematosus. A potential candidate cDNA clone for B700 has been obtained and is currently being sequenced and characterized in collaborative studies; preliminary evidence that this is the correct clone is strong since the protein encoded by that gene is recognized by the B700 specific antibodies following expression in a bacterial system.

Publications:

DeLeo AB, Hearing VJ, Vieira WD, Law LW. Serological characterization of a shared melanoma-associated antigen of mouse melanomas. Relationship to the melanoma-associated B700 glycoprotein. Melanoma Res 1991;1:133-140.

Farzaneh NK, Walden TL, Hearing VJ, Gersten DM. Comparison of the metabolic fate and distribution of B700, an albumin-like melanoma-specific antigen with serum albumin in naive and tumor-bearing mice. Int J Biochem 1991;23:1385-1391.

Farzaneh NK, Walden TL, Hearing VJ, Gersten DM. B700, an albumin-like melanoma-specific antigen, is a vitamin D-binding protein. Eur J Cancer 1991;27:1158-1162.

Gersten DM, Bijwaard KE, Walden TL, Hearing VJ. Serological demonstration of the albuminoid nature of the B700 murine melanoma antigen. Proc Soc Exp Biol Med 1991;197:310-316.

Gorelik E, Jay G, Kim M, Hearing VJ, DeLeo A, Herberman RB, McCoy JP. Effects of H-2K^b gene on expression of melanoma-associated antigen and lectin-binding sites on B16 melanoma cells. Cancer Res 1991;51:5212-5218.

Hearing VJ, Tsukamoto K. Enzymatic control of pigmentation in mammals. FASEB Journal 1991;5:2902-2909.

Tsukamoto K, Gersten DM, Law LW, Hearing VJ. Malignant melanoma. Relationship to parameters of differentiation. Melanoma Res 1991;1:223-230.

Gersten DM, Hearing VJ. Antigens of murine melanoma and their cross-species reactivity. Pathobiology 1992;60:49-56.

Gersten DM, Moody D, Vieira WD, Law LW, Hearing VJ. Production of mouse monoclonal antibodies to the murine melanoma antigen B700, and their anti-metastatic properties. Biochim Biophys Acta 1992;1038:109-114.

Hearing VJ. The metastatic phenotype, in Mechanisms of Drug Resistance in Oncology. In: Teicher BA, ed. New York: Marcel Dekker (in press).

Hearing VJ, King RA. Determinants of Skin Color: Melanocytes and Melanization, in Pigmentation and Pigmentary Abnormalities. In: Levine N, ed. New York: CRC Press (in press).

Hearing VJ, Tsukamoto K, Urabe K, Montague PM, Jackson I. Functional properties of cloned melanogenic proteins, in Proceedings of the Symposium on Molecular Biology of Pigment Cells. In: Takeuchi T, ed. Copenhagen: Munksgaard (in press).

Jackson IJ, Chambers DM, Tsukamoto K, Copeland NG, Gilbert DJ, Jenkins NA, Hearing VJ. A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse *slaty* locus. EMBO Journal 1992;11:527-535.

Shrayer D, Gersten DM, Maizel A, Wanebo H, Hearing VJ. B700 antigen as a component of an antimelanoma vaccine: formalinized extracellular antigens of murine melanoma (FECA). Pigment Cell Res (in press).

Tsukamoto K, Jackson I, Urabe K, Montague PM, Hearing VJ. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. EMBO Journal 1992;11:519-526.

Tsukamoto K, Jiménez M, Hearing VJ. The nature of tyrosinase isozymes. The Pigment Cell: From the Molecular to the Clinical Level. In: Mishima Y, ed. Munksgaard, Copenhagen, 1992;84-89.

Tsukamoto K, Palumbo A, d'Ischia M, Hearing VJ, Prota G. 5,6-Dihydroxyindole-2-carboxylic acid is incorporated in mammalian melanin, Biochem J (in press).

SUMMARY STATEMENT

ANNUAL REPORT
LABORATORY OF CELLULAR ONCOLOGY
DCBDC, NCI
October 1, 1991 through September 30, 1992

The Laboratory of Cellular Oncology plans and conducts fundamental research on the cellular and molecular basis of neoplasia. Investigators develop and employ tissue culture cell systems and animal models to study the induction and maintenance of benign and malignant neoplasia and reversal of the neoplastic state. They also elucidate structure-function correlations through detailed examination of individual genes which have been implicated in neoplasia. Spontaneous tumors from humans and other species are examined for the presence of exogenous genes or altered cellular genes. The main research results for the past year are as follows:

Tumor gene expression *in vitro* and *in vivo*

This project has studied ras encoded proteins.

We analyzed chimeras between ras and rap1A, which encodes a ras-like protein that can suppress ras-transformed cells. Chimeras that were discordant with respect to their sensitivity to GTPase acceleration were identified; some were sensitive to ras-GAP but resistant to NF1, and others were sensitive to cytoplasmic rap-GAP but resistant to membrane rap-GAP. Sensitivity of chimeras to ras-GAP and cytoplasmic rap-GAP was mediated by amino acids that are C-terminal to the effector region. In NIH 3T3 cells, chimeras carrying the p21ras effector region and sensitive only to ras-GAP or only to cytoplasmic rap-GAP were poorly transforming. Thus distinct amino acids of p21ras and p21rap1A mediate sensitivity to each of the proteins with GAP activity, and ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap1A, respectively, in NIH 3T3 cells.

We also identified and characterized the NF1 protein in mammalian cells. This protein is associated with another protein of very high molecular weight. In cell lines derived from malignant schwannomas that arose in patients with NF1 disease (von Recklinghausen's neurofibromatosis), the levels of NF1 protein were found to be very low and the proportion of ras in the active GTP-bound form was high, although there were no mutations in the ras protein. The results are consistent with NF1 being a tumor suppressor gene that negatively regulates ras. They also show that ras proteins can be activated by defective regulation, as well as by mutational activation.

Analysis of Papillomaviruses

Papillomaviruses (PVs) infect the epithelia of a wide variety 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. Our previous work has established that PVs encode three transforming genes: E5, E6 and E7. Others have shown that p53 binds the tumor suppressor protein p53 and induce its degradation in vitro. We have now determined that E6 decreases the in vivo half-life of p53 in human keratinocytes that express HPV16 E6 at least 8 fold. To examine the relevance of p53 inactivation to the biological activities of E6, we examined the ability of mutant p53, which specifically inactivates wild type p53, to functionally substitute for E6. It was able to substitute in cooperating with E7 to immortalize normal human keratinoctyes but not to transform NIH3T3 cells or trans-activate the adenovirus E1a promoter, indicating that E6 has both p53 dependent and independent activities.

Our previous studies have shown that BPV E5 induces the ligand independent activation of growth factor receptors. We have now constructed a series of PDGF and EGF receptor chimeras to

examine the receptor domains responsible for this activation. Our results indicate that the transmembrane domain of PDGFR is primarily responsible for its responsiveness to E5 while the intracellular domain of EGFR is required for its activation by E5. These results strongly suggest that E5 activates the two receptors by different mechanisms. We have begun to identify and characterize a cellular protein that specifically binds E5. Biochemical analysis and partial protein sequencing indicate that it is a new member of a family of proteins involved in intracellular protein trafficking

Analysis of the structural and immunogenic features of PVs has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, we have expressed the L1 major capsid proteins of BPV1 and HPV16 via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into PV virion-like structures. The self assembled BPV L1 resembled intact virions in being able to induce high titer neutralizing antiserum. These results indicate that L1 has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. This type of L1 preparation might be considered as a candidate for a vaccine to prevent PV infection.

Role of protein kinases in modulating cell growth and malignant transformation

The focus of this project is to better elucidate the possible involvement of transmembrane signal transmission systems in the regulation of cell growth and in malignant transformation. A new multiwell filtration assay was developed to determine protein kinase C (PKC) phosphotransferase and phorbol ester binding activities. This method is more rapid and is better suited for analyzing large numbers of samples than conventional methods. Exposure of cells to oxidant tumor promoters has been shown to directly modify PKC to convert it to an active form no longer dependent on Ca^{2+} and phospholipids. With this new method of assay, results indicate that short-term (15 to 30 min) pretreatment of cells with low (10 to 100 nM) concentrations of retinoic acid protects PKC from oxidants such as H_2O_2 and m-periodate. These results suggest that some of the anti-tumor promoter actions of retinoids may be through protection of PKC from oxidative modification.

Phosphate ion (Pi) uptake into NIH 3T3 cells was found to be differentially regulated by activation of PKC and of cAMP-dependent protein kinase (PKA). Activation of PKC resulted in the rapid (within min) stimulation of short-term (2 min) sodium-dependent Pi uptake, while activation of PKA resulted in the rapid inhibition of Pi transport. These results suggest that stimulation of PKC and PKA through hormone-induced production of diacylglycerol and cAMP, respectively, likely serves to rapidly regulate the intracellular level of Pi.

Previous studies have demonstrated a deficiency of PKA in fibroblasts and in erthrocytes isolated from patients with psoriasis. Retinoic acid treatment of psoriatic fibroblasts was shown to increase PKA to levels found in normal human fibroblasts. Results now indicate that the effects of retinoids on PKA are rapid. Treatment of isolated psoriatic erythrocytes with acitretin (a synthetic retinoid) for 15 min resulted in a significant increase in cAMP binding to the RI regulatory subunit of PKA. Oral administration of acitretin to psoriatic patients also resulted in a rapid (within 1 hr) increase in the ability of RI to bind cAMP. These data indicate that retinoids may act to rapidly modify PKA at the post-transcriptional level.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03663-16 LCO

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Tumor gene expression in vitro and in vivo

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

PI:	D. R. Lowy	Chief, Lab of Cellular Oncology	LCO NCI
OTHER:	J. E. DeClue	IRTA Fellow	LCO NCI
	K. Zhang	Visiting Associate	LCO NCI
	H. Cen	Visiting Fellow	LCO NCI
	M. Johnson	IRTA Fellow	LCO NCI
	W. D. Ju	Senior Staff Fellow	LCO NCI
	N. Tayebi	Predoctoral IRTA Fellow	LCO NCI
	A. G. Papageorge	Microbiologist	LCO NCI

COOPERATING UNITS *(if any)* University of Alberta, Edmonton, Canada, Dr. J. Stone
University Microbiology Instit., Copenhagen, Denmark, Drs. B.Willumsen &L.Beguinot
Hebrew University of Jerusalem, Jerusalem, Israel, Dr. A. Levitzki
Chiron Corporation, Emeryville, CA, Dr. F. McCormick

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	6.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Oncogene studies have involved ras encoded proteins. We analyzed chimeras between ras and rap1A, which encodes a ras-like protein that can suppress ras-transformed cells. Chimeras that were discordant with respect to their sensitivity to GTPase acceleration were identified; some were sensitive to ras-GAP but resistant to NF1, and others were sensitive to cytoplasmic rap-GAP but resistant to membrane rap-GAP. Sensitivity of chimeras to ras-GAP and cytoplasmic rap-GAP was mediated by amino acids that are C-terminal to the effector region. In NIH 3T3 cells, chimeras carrying the p21ras effector region and sensitive only to ras-GAP or only to cytoplasmic rap-GAP were poorly transforming. Thus, distinct amino acids of p21ras and p21rap1A mediate sensitivity to each of the proteins with GAP activity, and ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap1A, respectively, in NIH 3T3 cells.

We also identified and characterized the NF1 protein in mammalian cells. This protein is associated with another protein of very high molecular weight. In cell lines derived from malignant schwannomas that arose in patients with NF1 disease (von Recklinghausen's neurofibromatosis), the levels of NF1 protein were found to be very low and the proportion of ras in the active GTP-bound form was high, although there were no mutations in the ras protein. The results are consistent with NF1 being a tumor suppressor gene that negatively regulates ras. They also show that ras proteins can be activated by defective regulation, as well as by mutational activation.

We also used ras mutants to study ras in signal transduction. The results suggest that in NIH 3T3 cells the increase in GTP-bound ras protein in response to serum and growth factors may be mediated mainly by stimulated guanine nucleotide exchange. A region on the ras protein has been identified that may be required for the interaction between ras and exchange factors.

Other Professional Personnel:

W. C. Vass	Biologist	LCO NCI

Major findings:

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 and by performing structure-function analysis with mutants and chimeric genes. We have examined the influence of three proteins on ras - GAP, NF1, and raplA, as well as the role of ras in signal transduction. 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 collaboration with the laboratories of F. McCormick and B. Willumsen, we have previously found that GAP interacts with the effector region of ras protein. Since GAP interacts with this region of ras, GAP is a candidate for being the ras target in higher eukaryotes, in addition to its presumed function as a negative regulator of ras. 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. The rap1A gene encodes a ras-like protein that can suppress cells that are morphologically transformed by a highly oncogenic version of ras. rap-lA has its own GAP proteins (rap-GAP) that specifically accelerate its intrinsic GTPase activity.

After analyzing chimeras between ras and rap1A to genetically localize the segments that mediate their opposite function, we identified chimeric proteins that were discordant with respect to their sensitivity to GTPase acceleration; some were sensitive to ras-GAP but resistant to NF1, and others were sensitive to cytoplasmic rap-GAP but resistant to membrane rap-GAP. Sensitivity of chimeras to ras-GAP and cytoplasmic rap-GAP was mediated by amino acids that are C-terminal to the effector region. In NIH 3T3 cells, chimeras carrying the p21ras effector region and sensitive only to ras-GAP or only to cytoplasmic rap-GAP were poorly transforming. Thus distinct amino acids of p21ras and p21rap1A mediate sensitivity to each of the proteins with GAP activity, and sensitivity of the chimeric proteins to ras-GAP and cytoplasmic rap-GAP is specified by divergent residues located C-terminal to the effector domain, and ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap1A, respectively, in NIH 3T3 cells.

To identify NF1 protein in mammalian cells, we raised antisera to the catalytic region of NF1. These antisera show NF1 to be an evolutionarily conserved 280 kd cytoplasmic protein. Unlike GAP, NF1 protein is not phosphorylated on tyrosine in src transformed cells, suggesting that GAP and NF1 may be differentially regulated. By immunoprecipitation, a significant proportion of NF1 protein from cells is present as a high molecular weight complex that includes another protein that is even larger than NF1. We also examined three cell lines from malignant schwannomas that arose in patients with NF1 disease (von Recklinghausen's neurofibromatosis). All three lines had very low levels of NF1 protein, which was associated with high levels of GTP-bound normal ras protein in the cells. Expression of the catalytic domain of GAP induced morphological reversion of one of these lines and lowered its level of GTP-bound ras. These results support the hypothesis that NF1 is a tumor suppressor gene that in Schwann cells is a negative regulator of ras. These tumors show that ras can be activated by defective negative regulation, as well as by mutational activation.

To study ras in signal transduction in NIH 3T3 cells, we overexpressed two partially activated ras mutants, one that constitutively released guanine nucleotide and another that was partially resistant to GAP and NF1. Stimulation of cells with serum or PDGF increased the proportion of GTP on normal ras and the second mutant protein, but had no effect on the first mutant protein. The

proportion of GTP-bound to ras was higher in sub-confluent cells than in confluent cells, which correlated with confluent cells having higher GAP activity than sub-confluent cells. We conclude that stimulated guanine nucleotide exchange is responsible for most of the increase in GTP-bound to ras and speculate that loss of ras activity contributes to density dependent inhibition of cell growth. We also analyzed deletion mutations of normal ras which have been shown previously to be biologically active in the context of activating mutations in ras, to determine if there might be regions required for normal function that were dispensable for function by activated ras. We identified a region located on the surface of the protein that appears to be required for normal ras but not for mutationally activated ras. We conclude that this region may be involved in guanine nucleotide exchange.

Publications:

Lowy DR, Zhang K, DeClue JE, Willumsen BM: Regulation of p21 ras activity. Trends Genet 1991;7:346-51.

DeClue JE, Cohen BD, Lowy DR. Identification and characterization of the neurofibromatosis type 1 protein product. Proc Natl Acad Sci USA 1991;88:9914-8.

Willumsen BM, Vass WC, Velu TJ, Papageorge AG, Schiller JT, Lowy DR. The BPV E5 oncogene can cooperate with ras: identification of p21 amino acids critical for transformation by c-ras^{H} but not v-ras^{H}. Mol Cell Biol 1991;11:6026-33.

Zhang K, Papageorge AG, Martin P, Vass W, Olah Z, Polakis P, McCormick F, Lowy DR Heterogeneous amino acids in Ras and Rap1A specify sensitivity to GAP proteins. Science 1991;254:1630-4.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 1992;69:265-73.

Zhang K, Papageorge AG, Lowy DR. Mechanistic aspects of signaling through Ras in NIH 3T3 cells. Science 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08905-11 LCO

PERIOD COVERED: October 1, 1991 through September 30, 1992

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

Role of protein kinases in modulating cell growth and malignant transformation

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

PI:	W. B. Anderson	Research Chemist	LCO NCI
OTHER:	Z. Olah	Visiting Associate	LCO NCI
	J. W. Karaszkiewicz	Biotechnology Fellow	LCO NCI
	C. Lehel	Visiting Fellow	LCO NCI
	A. Ferrier	IRTA Fellow	LCO NCI
	J. A. Strasburger	Microbiologist	LCO NCI

COOPERATING UNITS (if any)

see next page

LAB/BRANCH: Laboratory of Cellular Oncology

SECTION:

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	5.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The focus of this project is to better elucidate the possible involvement of transmembrane signal transmission systems in the regulation of cell growth and in malignant transformation. A new multiwell filtration assay was developed to determine protein kinase C (PKC) phosphotransferase and phorbol ester binding activities. This method is more rapid and is better suited for analyzing large numbers of samples than conventional methods. Exposure of cells to oxidant tumor promoters has been shown to directly modify PKC to convert it to an active form no longer dependent on Ca^{2+} and phospholipids. With this new method of assay, results indicate that short-term (15 to 30 min) pretreatment of cells with low (10 to 100 nM) concentrations of retinoic acid protects PKC from oxidants such as H_2O_2 and m-periodate. These results suggest that some of the anti-tumor promoter actions of retinoids may be through protection of PKC from oxidative modification.

Phosphate ion (Pi) uptake into NIH 3T3 cells was found to be differentially regulated by activation of PKC and of cAMP-dependent protein kinase (PKA). Activation of PKC resulted in the rapid (within min) stimulation of short-term (2 min) sodium-dependent Pi uptake, while activation of PKA resulted in the rapid inhibition of Pi transport. These results suggest that stimulation of PKC and PKA through hormone-induced production of diacylglycerol and cAMP, respectively, likely serves to rapidly regulate the intracellular level of Pi.

Raf-1 protein kinase (Raf-1 PK) levels and activity were found to be elevated in human radiation resistant squamous carcinoma cells. Treatment of these cells with antisense c-raf-oligodeoxyribonucleotides (ODNS) was found to decrease Raf-1 PK activity in a concentration (20-40 mM)- and time (12-36 hrs)-dependent manner. This experimental approach should be useful in determining the possible role of Raf-PK in radiation resistance.

Previous studies have demonstrated a deficiency of PKA in fibroblasts and in erthrocytes isolated from patients with psoriasis. Retinoic acid treatment of psoriatic fibroblasts was shown to increase PKA to levels found in normal human fibroblasts. Results now indicate that the effects of retinoids on PKA are rapid. Treatment of isolated psoriatic erythrocytes with acitretin (a synthetic retinoid) for 15 min resulted in a significant increase in cAMP binding to the RI regulatory subunit of PKA. Oral administration of acitretin to psoriatic patients also resulted in a rapid (within 1 hr) increase in the ability of RI to bind cAMP. These data indicate that retinoids may act to rapidly modify PKA.

Cooperating Units:

LVC, NCI, FCRDC, Dr. U. Rapp
Dept. Pharmacol. & Nutrition, USC School of Medicine, Los Angeles, CA, Dr. R. Gopalakrishna
Lab.of Physiopath. Development, Paris, France, Drs. D. Evain-Brion, and F. Raynaud
Dept. Radiation Med., Georgetown Univ. School of Medicine, Washington, DC, Dr. U. Kasid

Major Findings:

1. Protein Kinase C. Protein kinase C (PKC) is a calcium-activated, phospholipid-dependent serine threonine protein kinase of fundamental importance in transmembrane signal transmission. 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. To factilitate the study of PKC a new method was developed to measure PKC activities. With this method both the incubations and filtrations necessary to assay PKC activity and phorbol ester tumor promoter binding to PKC can be carried out in multiwell plates with fitted filtration discs. This method is significantly more rapid and more convenient for analyzing large numbers of samples than conventional methods.

Studies in collaboration with Dr. R. Gopalakrishna have established that oxidative modification may be an important regulatory parameter of PKC. Oxidant tumor promoters such as m-periodate and benzoyl peroxide were found to directly modify the regulatory domain of PKC to generate an active kinase no longer regulated by Ca^{2+} or phospholipids. Since retinoids (vitamin A derivatives) have been shown to antagonize the actions of tumor promoters, studies were carried out to determine if retinoids can inhibit the oxidative modification of PKC induced by tumor promoters. Prior treatment of intact cells with retinoic acid for a short time period (15 to 60 min) was found to decrease the oxidative activation of PKC caused by treatment of the cells with oxidants such as hydrogen peroxide and m-periodate. Results of experiments with isolated PKC showed that retinoids can act directly to protect PKC from oxidative modification induced by oxidants, although high (1-10μM) concentrations of retinoid are required. In contrast, with intact cells only low (submicromolar) concentrations of retinoids are required to protect PKC from oxidants. These results suggest that some of the anti-tumor promoter actions of retinoids may be mediated, in part, by inhibiting the oxidative activation of PKC induced oxidant tumor promoters.

Reports by others have shown that phosphate ion (Pi) uptake into the cell can serve as an important regulatory mechanism for intracellular metabolism and cell proliferation. Since sodium-dependent Pi transport has been shown to be regulated by various receptors and second messengers, studies were carried out to characterize the differential regulation of Pi transport by the PKC and cAMP-dependent protein kinase (PKA) signalling pathways in NIH 3T3 cells. Activation of PKC by phorbol ester (TPA) or diacylglycerol analogue (OAG) treatment of these cells resulted in a rapid (within 2-5 min) stimulation of short-term (2 min) Pi transport. Conversely, preincubation of the cells with forskolin or cholera toxin to stimulate adenylate cyclase, and treatment with 8-bromo cAMP, to activate PKA resulted in a decrease in Na^+/Pi-uptake. Stimulation of Na^+/Pi-transport by PKC activation was found to be due to an increase in the affinity of transporter for Pi, which inhibition of Pi-uptake by PKA activation was found to be due to a decrease in the affinity for Pi. These results indicate that Na^{2+}/Pi-uptake in NIH 3T3 cells is differentially regulated by activators of PKC (stimulation) and PKA (inhibition) as a consequence of changes in the affinity for Pi.

2. Raf protein kinase. Since PKC plays a major role in cell growth regulation and tumor promotion, studies have been aimed at identifying proteins and activities modified by PKC to, in turn, transduce a signal to other components of the cell, including the nucleus. One activity found

to be modified by initial activation of PKC is the Raf-1 protein kinase (Raf-1 PK), encoded by a member of the raf proto-oncogene family. Studies also indicate that Raf-1 PK levels and phosphotransferase activity are elevated in radiation resistant human squamous carcinoma cells. To better understand the possible role of Raf-1 PK in radiation resistance, these cells were treated with antisense c-raf-1 oligodeoxyribonucleotides (ODNS) (25-mers) in an attempt to block the synthesis of Raf-1 PK. Exposure of radiation resistant squamous carcinoma cells to these antisense ODNS's was found to significantly decrease Raf-1 PK activity in a concentration (20-40 μM)- and time (12-36h)- dependent manner. These results indicate that AS c-raf-1 ODNS's are capable of decreasing Raf-1 PK in these cells, and that this approach should be useful in studies on the functional role of this kinase in radiation resistant tumors.

3. Retinoids in mediating cell growth and tumor promotion. In collaboration with Dr. D. Evain-Brion, studies were continued to better understand the role of decreased PKA in the hyperproliferative skin disease psoriasis, and to study the mechanism by which retinoic acid treatment of psoriatic patients increases the low level of PKA toward normal. Results of new studies indicate that the effect of retinoids on PKA in psoriatic cells is very rapid. Oral administration of acitretin (a synthetic retinoid) to psoriatic patients induced a rapid (within 1 hr) increase in the ability of the RI subunit to bind cAMP in erythrocytes prepared from these patients. In addition, short-term exposure (15 min) of psoriatic erythrocytes to acitretin also promoted an increase in cAMP binding to the RI regulatory protein of PKA. These results suggest that acitretin may act to rapidly modify PKA (the RI subunit) at the post-transcriptional level and this may reflect, in part, on the mechanism of action and efficacy for the in vivo therapeutic effects of retinoids.

Publications:

Takahashi N, Liapi C, Anderson WB, Breitman TR. Retinoylation in HL60 cells of the cAMP-binding regulatory subunits of type I and type II cAMP-dependent protein kinases. Archives Biochem Biophys 1991;290:293-302.

Kiss Z, Crilly KS, Rossi MA, Anderson WB. Selective inhibition by 4-hydroxynonenal of sphingosine-stimulated phospholipase D in NIH 3T3 cells. Biochim Biophys Acta 1992;1124:300-2.

Lee SA, Karaszkiewicz JW, Anderson WB. Elevated level of nuclear protein kinase C in multidrug-resistant MCF-7 human breast carcinoma cells. Cancer Res 1992; in press.

Gopalakrishna R, Chen ZH, Gundimeda U, Wilson JC, Anderson WB. Rapid filtration assays for protein kinase C activity and phorbol ester binding using multiwell plates with fitted filtration discs. Anal Biochem 1992; in press.

Raynaud F, Gerbaud P, Bouloc A, Gorin I, Anderson WB, Evain-Brion D. Rapid effect of the synthetic retinoid acitretin on psoriatic erythrocytes to increase 8 azido cyclic AMP binding to the RI regulatory subunit: In vivo and in vitro studies. J Invest Dermatol 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09052-04 LCO

PERIOD COVERED: October 1, 1991 through September 30, 1992

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

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

PI:	J. T. Schiller	Research Microbiologist	LCO NCI
OTHER:	D. R. Lowy	Chief, LCO	LCO NCI
	S. A. Sedman	IRTA Fellow	LCO NCI
	B. D. Cohen	IRTA Fellow	LCO NCI
	R. Kirnbauer	Special Volunteer	LCO NCI
	R. M. Melillo	Visiting Fellow	LCO NCI
	N. L. Hubbert	Microbiologist	LCO NCI
	J. V. Taub	Bio Lab Technician	LCO NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Cellular Oncology

SECTION:

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

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Papillomaviruses (PVs) infect the epithelia of a wide variety 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. Our previous work has established that PVs encode three transforming genes: E5, E6 and E7. Others have shown that E6 binds the tumor suppressor protein p53 and induce its degradation in vitro. We have now determined that E6 decreases the in vivo half-life of p53 in human keratinocytes that express HPV16 E6 at least 8 fold. To examine the relevance of p53 inactivation to the biological activities of E6, we examined the ability of mutant p53, which specifically inactivates wild type p53, to functionally substitute for E6. It was able to substitute in cooperating with E7 to immortalize normal human keratinoctyes but not to transform NIH3T3 cells or trans-activate the adenovirus E1a promoter, indicating that E6 has both p53 dependent and independent activities.

Our previous studies have shown that BPV E5 induces the ligand independent activation of growth factor receptors. We have now constructed a series of PDGF and EGF receptor chimeras to examine the receptor domains responsible for this activation. Our results indicate that the transmembrane domain of PDGFR is primarily responsible for its responsiveness to E5 while the intracellular domain of EGFR is required for its activation by E5. These results strongly suggest that E5 activates the two receptors by different mechanisms. We have begun to identify and characterize a cellular protein that specifically binds E5. Biochemical analysis and partial protein sequencing indicate that it is a new member of a family of proteins involved in intracellular protein trafficking

Analysis of the structural and immunogenic features of PVs has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, we have expressed the L1 major capsid proteins of BPV1 and HPV16 via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into PV virion-like structures. The self assembled BPV L1 resembled intact virions in being able to induce high titer neutralizing antiserum. These results indicate that L1 has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. This type of L1 preparation might be considered as a candidate for a vaccine to prevent PV infection.

Major Findings:

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. However, in some cases, the lesions induced by certain PVs undergo malignant progression. There is a strong association between malignant progression of human genital lesions and certain HPV types, most frequently HPV16. PVs encode three transforming genes: E6, E7 and E5. A major goal of the laboratory has been to elucidate the mechanisms by which the protein products of the these genes induce cell proliferation and transformation.

1. p53 metabolism in E6 containing cells. It has previously been determined that in vitro synthesized HPV16 E6 binds the tumor suppressor protein p53 and induces its in vitro degradation. Since it has not been determined if E6 influences the metabolism of p53 in vivo, we have measured the half-life of newly synthesized p53 in human keratinocytes before and after the introduction of HPV16 E6. The p53 in normal human keratinocytes had a suprisingly long half-life of 4 hr while the E6 expressing derivatives had half-lives of less than 30 min. In the absence of E7, the E6 expressing cells rapidly senesced indicating that rapid degradation of p53 is not sufficient to induce immortalization. In many E6 plus E7 immortalized lines, the total amount of p53, as measured by immunoblotting, was not significantly reduced. These results suggest that E6 may preferentially degrade newly synthesized p53 and therefore that this pool may be preferentially active in growth suppression.

2. The relationship between p53 inactivation and the activities of E6. We have previously identified three biological activities of HPV16 E6: transformation of NIH3T3 cells, trans-activation of the adenovirus E2 promoter, and cooperation with E7 to immortalize normal human keratinocytes. To determine the role of p53 inactivation in these three activities, we tested the ability of mutant p53, which specifically inhibits the activity of wild type p53, to fuctionally substitute for E6 in the three assays. Both human and mouse mutant p53 could replace E6 in the keratinocyte immortalization assay but they did not have the transforming and trans-activating activities of E6. It therefore appears that functional inactivation of p53 is important for E6 induced immortalization but that a separable E6 function is responsible for the other two activities.

3. The E5 responsive domains of PDGF and EGF receptors. We have previously shown that E5 induces the ligand independent activation of growth factor receptors. To determine the domains responsible for E5 induced activation, we have made a series of chimeras between PDGFR and EGFR, separating the molecules into extracellular, transmembrane and intracellular domains. Using CHO cells, which lack both receptors, we determined that E5 activation of the PDGFR requires the PDGFR transmembrane domain. EGFR were not activated by E5 in CHO cells. However, in NIH3T3 cells, which contain E5 activated PDGFR, the EGFR was activated by E5. Surprisingly, only the intracellular domain was specifically required for activation. These results demonstrate that E5 activates the two receptors by different mechanisms and suggest that activation of EGFR may be secondary to activation of PDGFR. However, it is unlikely that EGFR activation is due simply to trans-phosphorylation by PDGFR, since activation of PDGFR by ligand did not activate co-expressed EGFR.

4. Identification and characterization of an E5 binding cellular protein. The identification of E5 associated cellular proteins has been hampered by its small size (44 amino acids) and high degree of hydrophobicity. To partially overcome this problem, we have expressed the C-terminal domain of E5, which is hydrophilic and required for E5 transforming activity, as a fusion protein linked to bacterial glutathione-S-transferase (GST). The GST-E5 was purified on a glutathione containing column, and the bound protein incubated with cellular extracts. A single cellular protein specifically bound the wild type E5 peptide but not the corresponding peptide from a non-

transforming mutant of E5. This cellular protein, which is associated with a strong in vitro kinase activity, was preparatively purified and subjected to biochemical and partial sequence analysis. It appears to be a new member of a family of cellular proteins implicated in intracellular protein trafficking. This result is interesting in view of our previous finding that E5 inhibits the down regulation of activated EGFR.

5. Self-assembly of the PV L1 into particles that are morphologically and immunologically similar to native virions. Analysis of the structural and immunogenic features of papillomavirus virions has been hampered by the inability to propagate the viruses in cultured cells. To partially overcome this handicap, we have expressed the L1 major capsid protein of BPV1 and HPV16 in insect cells via baculovirus vectors. The L1 proteins were expressed at high levels and assembled into structures that closely resembled PV virions. In contrast to bacterially derived L1, the self-assembled L1 mimicked intact BPV virions in being able to induce high titer neutralizing rabbit antisera. These results indicate that L1 protein has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. This type of L1 preparation might be considered as a candidate for a serological test to measure the prevalence of neutralizing antibodies and for a vaccine to prevent PV infection.

Publications:

Sedman SA, Barbosa MS, Vass WC, Hubbert NL, Hass JA, Lowy DR, Schiller JT. The full-length E6 protein of HPV16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in cutlture. J Virol 1991;65:4860-6.

Sedman SA, Hubbert NL, Vass WC, Lowy DR, Schilller JT. Mutant p53 can substitute for HPV16 E6 in immortalization of human keratinocytes but does not have E6-associated trans-activation or transforming activity. J Virol 1992;66:4201-8.

Hubbert NL, Sedman SA, Schiller JT. HPV16 E6 increases the degradation rate of p53 in human keratinocytes. J Virol 1992; in press.

Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1991 to September 30, 1992

The genetic basis of human renal cell carcinoma has been the major focus of the research effort of the Cellular Immunity Section. We have continued to study hereditary and sporadic renal cell carcinomas and continued the isolation and characterization of new probes from human chromosome 3.

Von Hippel-Lindau disease (VHL) is an autosomal dominant, multisystem neoplastic disorder. We have located the VHL gene in a 6-8 cM interval between RAF1 and D3S18. Last year we reported that D3S601, a marker located between RAF1 and D3S18, does not show recombination with VHL.

To locate VHL more precisely, this year we prepared a long range restriction map aound VHL. The map covers 2.4 megabases. We examined 75 unrelated individuals for evidence of a rearrangment in the VHL gene region. One family was identified in which affected individuals appear to have a 200 kilobase deletion in the VHL gene region.

Yeast artificial chromosomes containing human DNA inserts were isolated for the markers which are located on either side of VHL and for D3S601. About 500 kilobases of DNA around D3S601 have been cloned.

One family was identified in which renal cell carcinoma is inherited as an autosomal dominant trait. The gene which produces renal cell carcinoma in this family appears to be distinct from the VHL gene. Ten individuals in this family have been shown to have renal cell carcinoma.

The translocation breakpoint in the t3;8 RCC family was mapped between D3S1187 and PTPγ loci in an ~2cM interval.

While supported by a grant from the National Center for Human Genome Research, we have assisted in the preparation of a high resolution genetic map of human chromosome 3. To date, 122 loci have been placed in the short arm of chromosome 3 and 40 loci have been placed on the long arm of chromosome 3.

The Immunopathology Section has continued studies of three different pro-inflammatory proteins. Partial sequences of Macrophage Stimulating Protein (MSP), isolated from human serum, showed that MSP belongs to the group of kringle proteins that includes hepatocyte growth factor and several proteolytic enzymes of the coagulation system (prothrombin, plasminogen, plasminogen activator, urokinase, clotting factors IX and X). After detecting MSP by ELISA in culture fluid of the human hepatoma cell line, HepG2, we cloned the cDNA for MSP from this cell line. The cDNA comprises 2300 bp with a single open reading frame that encodes for 701 amino acids. MSP has 4 kringles, among related proteins its sequence is most similar to hepatocyte growth factor. In addition to the MSP cDNA, we found several other clones with different types of insertion or deletion. By Northern blot, MSP cDNA strongly hybridized to several mRNA species, which is probably due to alternative splicing. The cDNA hybridized to mRNAs from liver, kidney, and

pancreas. The strongest hybridization of the cDNA to liver mRNA suggests that MSP is largely produced by liver. By in situ hybridization, the MSP gene has been mapped to chromosome 1. Hybridization of human MSP cDNA to animal genomic DNAs suggests that MSP is highly conserved among species. The supernatants of COS-7 cells transfected with MSP cDNA contained MSP activity and the expressed MSP was detected by immunoprecipatation with an antibody against native MSP.

Neutrophil attractant protein-1 (NAP-1 or IL-8) and monocyte chemoattractant protein-1 (MCP-1) are relatively specific attractants for human leukocytes. NAP-1 attracts neutrophils, but not monocytes; the converse is true for MCP-1. If selective secretion of these attractants accounts for specificity of leukocyte infiltrates, there should be conditions in which one is secreted without the other. With this in mind, secretory responses to LPS by elutriated monocytes, cultured monocytes and bronchoalveolar lavage (BAL) macrophages were compared. BAL macrophages ($2x10^6$/ml) secreted large amounts of NAP-1 in response to LPS (153 $\pm$ 25 nM), whereas LPS caused no increse in MCP-1 above a low baseline level of 0.5 $\pm$ 0.2 nM. In contrast, monocytes that matured in tissue culture had a 23-fold higher capacity for MCP-1 secretion than the reference freshly elutriated population, compared to only a 3-fold increase for NAP-1. If the cultured monocytes are a model of tissue macrophages (in contrast to blood monocytes or BAL macrophages), the data may reflect an in vivo pattern in which tissue macrophages produce MCP-1 to attract blood monocytes to aninflammatory site. BAL macrophages, at the interface between host and outside world, appear to be programmed for predominantly NAP-1 secretion to recruit neutrophils in an acute response to invasion by pathogens. We also studied the capacity of other cytokines to stimulate monocyte secretion of NAP-1 or MCP-1. Significant stimulation occurred with IL-1α, and the combination of IL-2 and IFNγ (which makes monocytes responsive to IL-2). IL-4, which has both stimulating and inhibitory effects on human monocytes, profoundly decreased LPS-induced monocyte secretion of both NAP-1 and MCP-1.

We reported last year that serum of normal human subjects contains a complex of NAP-1 and IgG. Gel filtration of immunoaffinity purified NAP-1 IgG showed that the bulk of the complex comprised a single IgG. Recent studies showed that binding between NAP-1 and antibody is strong,since 8 M srea at neutral or alkaline pH did not release NAP-1. However, at pH 2.0 in 9 M urea about 15% of the total NAP-1 could be dissociated from the complex. NAP-1-IgG was detected in 18 of 26 sera from normal humans. The mean serum concentration was 58 ng of complex NAP-1 per ml, with an SEM of 16 and a range from undetectable to 247 ng/ml. NAP-1-IgG concentrations in paired sera drawn at a 1 month interval were remarkably constant. Using an ELISA for free NAP-1 with a detection limit of 200pg/ml, we found no free NAP-1 in the 26 sera. Free anti-NAP-1 autoantibody was found in 9 of 26 sera by direct ELISA. IgG anti-NAP-1 of all 9 sera was polyclonal, comprising both kappa and lambda isotypes; predominant subclasses were IgG2 and IgG3. This is in striking contrast to the mainly IgG1 subclass for normal human pooled IgG. NAP-1-IgG did not compete with ^{125}I-NAP-1 for binding to neutrophils, which suggests that IgG anti-NAP-1 is a molecular trap that prevents binding of NAP-1 to neutrophils after it diffuses from production sites into the circulation. We have also found MCP-1-IgG and free IgG anti-MCP-1 in normal human serum. We had the opportunity to measure these proteins in sera of human subjects who received a single intravenous injection of LPS (bacterial endotoxin). LPS caused a rise

to a peak within 2-3 hrs in serum concentrations of free NAP-1 and MCP-1, followed by an almost equally rapid fall toward baseline levels by about 5 hrs post injection. MCP-1 concentration in sera from the 11 subjects rose from a pre-LPS value of 8 $\pm$ 4 pM (SEM) to a peak of 320 $\pm$ 51 pM. In 10 of the 11 subjects, free IgG autoantibody to MCP-1 decreased from a pre-LPS value of 1820 $\pm$ 660 pM to a mean low of 53 $\pm$ 7% of the control. The decrease in concentration of free antibody as MCP-1 serum concentration rose during the first 2-3 hours after LSP challenge probably reflects depletion as the blood of antigen combined with antibody to form immune complexes. However, since we observed no consistent change in concentrations of immune complex, the means by which these complexes are cleared in the LPS-challenged subjects remains unknown.

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

PROJECT NUMBER
Z01 CB 08575-19 LIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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.

Partial sequences of Macrophage Stimulating Protein (MSP), isolated from human serum, showed that MSP belongs to the group of kringle proteins that includes hepatocyte growth factor and several proteolytic enzymes of the coagulation system (prothrombin, plasminogen, plasminogen activator, urokinase, clotting factors IX and X). After detecting MSP by ELISA in culture fluid of the human hepatoma cell line, HepG2, we cloned the cDNA for MSP from this cell line. The cDNA comprises 2300 bp with a single open reading frame that encodes for 701 amino acids. MSP has 4 kringles, among related proteins its sequence is most similar to hepatocyte growth factor. In addition to the MSP cDNA, we found several other clones with different types of insertion or deletion. By Northern blot, MSP cDNA strongly hybridized to several mRNA species, which is probably due to alternative splicing. The cDNA hybridized to mRNAs from liver, kidney, and pancreas. The strongest hybridization of the cDNA to liver mRNA suggests that MSP is largely produced by liver. By in situ hybridization, the MSP gene has been mapped to chromosome 1. Hybridization of human MSP cDNA to animal genomic DNAs suggests that MSP is highly conserved among species. The supernatants of COS-7 cells transfected with MSP cDNA contained MSP activity and the expressed MSP was detected by immunoprecipatation with an antibody against native MSP.

Neutrophil attractant protein-1 (NAP-1 or IL-8) and monocyte chemoattractant protein-1 (MCP-1) are relatively specific attractants for human leukocytes. NAP-1 attracts neutrophils, but not monocytes; the converse is true for MCP-1. If selective secretion of these attractants accounts for specificity of leukocyte infiltrates, there should be conditions in which one is secreted without the other. With this in mind, secretory responses to LPS by elutriated monocytes, cultured monocytes and bronchoalveolar lavage (BAL) macrophages were compared. BAL macrophages ($2x10^6$/ml) secreted large amounts of NAP-1 in response to LPS (153 $\pm$ 25 nM), whereas LPS caused no increase in MCP-1 above a low baseline level of 0.5 $\pm$ 0.2 nM. In contrast, monocytes that matured in tissue culture had a 23-fold higher capacity for MCP-1 secretion than the reference freshly elutriated population, compared to only a 3-fold increase for NAP-1. If the cultured monocytes are a model of tissue macrophages (in contrast to blood monocytes or BAL macrophages), the data may reflect an in vivo pattern in which tissue macrophages produce MCP-1 to attract blood monocytes to an inflammatory site. BAL macrophages, at the interface between host and outside world, appear to be programmed for predominantly NAP-1 secretion to recruit neutrophils in an acute response to invasion by pathogens. We also studied the capacity of other cytokines to stimulate monocyte secretion of NAP-1 or MCP-1. Significant stimulation occurred with IL-1α, and the combination of IL-2 and IFNγ (which makes monocytes responsive to IL-2). IL-4, which has both stimulating and inhibitory effects on human monocytes, profoundly decreased LPS-induced monocyte secretion of both NAP-1 and MCP-1.

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was found in 9 of 26 sera by direct ELISA. IgG anti-NAP-1 of all 9 sera was polyclonal, comprising both kappa and lambda isotypes; predominant subclasses were IgG2 and IgG3. This is in striking contrast to the mainly IgG1 subclass for normal human pooled IgG. NAP-1-IgG did not compete with ^{125}I-NAP-1 for binding to neutrophils, which suggests that IgG anti-NAP-1 is a molecular trap that prevents binding of NAP-1 to neutrophils after it diffuses from production sites into the circulation. We have also found MCP-1-IgG and free IgG anti-MCP-1 in normal human serum. We had the opportunity to measure these proteins in sera of human subjects who received a single intravenous injection of LPS (bacterial endotoxin). LPS caused a rise to a peak within 2-3 hrs in serum concentrations of free NAP-1 and MCP-1, followed by an almost equally rapid fall toward baseline levels by about 5 hrs post injection. MCP-1 concentration in sera from the 11 subjects rose from a pre-LPS value of 8 $\pm$ 4 pM (SEM) to a peak of 320 $\pm$ 51 pM. In 10 of the 11 subjects, free IgG autoantibody to MCP-1 decreased from a pre-LPS value of 1820 $\pm$ 660 pM to a mean low of 53 $\pm$ 7% of the control. The decrease in concentration of free antibody as MCP-1 serum concentration rose during the first 2-3 hours after LSP challenge probably reflects depletion as the blood of antigen combined with antibody to form immune complexes. However, since we observed no consistent change in concentrations of immune complex, the means by which these complexes are cleared in the LPS-challenged subjects remains unknown.

Publications

Leonard E, Sylvester I, and Yoshimura T. Assay of neutrophil attractant protein-1 (NAP-1). In Current Protocols in Immunology. Wiley, New York. Coligan JE et al, eds. 1991.

Yoshimura T, Takeya M, Takahashi K, Kuratsu J and Leonard EJ. Production and characterization of mouse monoclonal antibodies against human monocyte chemoattractant protein (MCP-1). J Immunol 1991;147:2229-2233.

Yoshimura T and Leonard EJ. Monocyte chemoattractant protein-1 (MCP-1): Structure and Function. In Cytokines, Vol. 4. Edited by M. Baggiolini and C. Sorg. Karger, Basel. 1992:131-152.

Yla-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, Witztum JL and Steinberg D. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci USA 1992;88:5252-5256.

Rovin BH, Yoshimura T and Tan L. Cytokine-induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. J Immunol 1992; 148:2148-2153.

Sylvester I, Yoshimura T, Sticherling M, Schroder JM, Ceska M, Peichl P, Leonard J. NAP-1-IgG immune complexes and free anti-NAP-1 antibody in normal human serum. J Clin Invest, in press.

T. Yoshimura. cDNA cloning of guinea pig monocyte chemoattractant protein-1 and expression of the recombinant protein. J Immunol, in press.

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

PROJECT NUMBER
Z01 CB 08577-07 LIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Restriction fragment length polymorphisms in normal and neoplastic tissues

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

CO-PI: B. Zbar and M. Lerman

Other: F. Latif — Visiting Scientist
M. Yao — Visiting Fellow
F.W. Zhou — Visiting Scientist

COOPERATING UNITS (if any)

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 B
☐ (a2) Interviews

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

The major goal of the research effort was to precisely locate the von Hippel-Lindau disease gene. Previous studies from our laboratory indicated that VHL is located in a 6-8 cM interval between RAF1 and D3S18. D3S601, located between RAF1 and D3S18, has not shown any recombination with VHL. A long range restriction map around VHL has been prepared by pulsed field gel electrophoresis. The physical map is centered around D3S601. One family has been identified in which affected members appear to have a 200 kb deletion in the VHL gene area. We have isolated YAC clones for all markers close to VHL and are in the process of cloning the region by YAC-based chromosome walking.

Major findings:

We constructed a long range restriction map around VHL by pulsed gield gel electrophoresis. The map, 2.4 megabases in size, is centered around D3S601. One family has been identified in which affected members appear to have a 200 kb deletion in the VHL gene area.

An American family has been identified with "pure" inherited renal cell carcinoma. Ten family members have had renal cell carcinoma. Histologically, the tumors are adenocarcinomas of the kidney with papillary features. No translocations or rearrangements were detected in the constitutional tissues of this family by cytogenetics. Preliminary results indicate that the gene responsible for renal cell carcinoma in this family is distinct from VHL.

Publications:

Latif F, Glenn GM, Tory K, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 46-100 (D3S641). Nucleic Acids Res. 1991;19:1170.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 11-95 (D3S730). Nucleic Acids Res. 1991;19:2801.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 28-62 (D3S626). Nucleic Acids Res. 1991;19:2800.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 30-62′ (D3S606). Nucleic Acids Res. 1991;19:2798.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 35-53 (D3S731). Nucleic Acids Res. 1991;19:2801.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 46-35′ (D3S636). Nucleic Acids Res. 1991;19:2799.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 17-58 (D3S735). Nucleic Acids Res. 1991;19:2799.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 25-55 (D3S736). Nucleic Acids Res. 1991;19:2797.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 41-10 (D3S737). Nucleic Acids Res. 1991;19:2797.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 46-95 (D3S590). Nucleic Acids Res. 1991;19:2797.

Latif F, Glenn GM, Brauch H, Delisio J, Hampsch K, Orcutt ML, Zbar B, Lerman MI. A new polymorphic probe on chromosome 3p: LIB 29-48 (D3S725). Nucleic Acids Res. 1991;19:2800.

Wei M, Latif F, Tory K, et al. A new polymorphic probe on chromosome 3p: LIB 18-88 (D3S1093). Nucleic Acids Res. 1991;19:4575.

Li H, Latif F, Tory K, et al. A new polymorphic probe on chromosome 3p: LIB 17-11 (D3S1092). Nucleic Acids Res. 1991;19:4564.

Delisio J, Latif F, Glenn GM, et al. A new polymorphic probe on chromosome 3p: LIB 50-50′ (D3S1099). Nucleic Acids Res. 1991;19:4568.

Delisio J, Latif F, Glenn GM, et al. A new polymorphic probe on chromosome 3p: LIB 46-27′ (D3S635). Nucleic Acids Res. 1991;19:4564.

Delisio J, Latif F, Glenn GM, et al. A new polymorphic probe on chromosome 3p: LIB 4-59 (D3S575). Nucleic Acids Res. 1991;19:4568.

Latif F, Glenn GM, Geil L, et al. A new polymorphic probe on chromosome 3p: LIB 27-98" (D3S1096). Nucleic Acids Res. 1991;19:4565.

Latif F, Glenn GM, Geil L, et al. A new polymorphic probe on chromosome 3p: LIB 45-86 (D3S633). Nucleic Acids Res. 1991;19:4565.

Latif F, Glenn GM, Geil L, et al. A new polymorphic probe on chromosome 3p: LIB 39-63 (D3S616). Nucleic Acids Res. 1991;19:4566.

Latif F, Glenn GM, Geil L, et al. A new polymorphic probe on chromosome 3p: LIB 27-77 (D3S603). Nucleic Acids Res. 1991;19:4566.

Latif F, Glenn GM, Geil L, et al. A new polymorphic probe on chromosome 3p: LIB 13-67 (D3S591). Nucleic Acids Res. 1991;19:4567.

Geil L, Latif F, Yao M, et al. A new polymorphic probe on chromosome 3p: LIB 36-68 (D3S615). Nucleic Acids Res. 1991;19:4569.

Latif F, Glenn GM, Geil L, et al. A new polymorphic probe on chromosome 3p: LIB 37-96′ (D3S1192). Nucleic Acids Res. 1991;19:4567.

Latif F, Tory K, Geil L, Zbar B, Lerman MI. A new DNA marker, D3S640, identifies a DraI, HindIII, and TaqI polymorphism on chromosome 3p. Nucleic Acids. Res. 1991;19:6660.

Latif F, Tory K, Geil L, Zbar B, Lerman MI. A new DNA marker, D3S743, identifies a MspI polymorphism on chromosome 3p. Nucleic Acids. Res. 1991; 19:6660.

Latif F, Tory K, Geil L, Zbar B, Lerman MI. A new DNA marker, D3S742, identifies a MspI and a TaqI polymorphism on chromosome 3p. Nucleic Acids. Res.1991;19: 6659.

Latif F, Tory K, Geil L, Zbar B, Lerman MI. A new DNA marker, D3S733, identifies a HindIII polymorphism on chromosome 3p. Nucleic Acids. Res. 1991;19:6659.

Latif F, Tory K, Geil L, Zbar B, Lerman MI. A new DNA marker, D3S740, identifies a MspI polymorphism on chromosome 3p. Nucleic Acids. Res. 1991; 19:6658.

Latif F, Tory K, Geil L, Zbar B, Lerman MI. A new DNA marker, D3S754, identifies a HindIII polymorphism on chromosome 3p. Nucleic Acids. Res. 1991;19:6658.

Anglard P, Trahan E, Liu S, Latif F, Merino M, Lerman MI, Zbar B, Linehan WM. Molecular and cellular characterization of human renal cell carcinoma cell lines. Can. Res. 1992;52:348-356.

Gnarra JR, Anglard P, Latif F, Tory K, Glenn G, Lerman MI, Zbar B, Linehan WM. Molecular studies of sporadic and familial renal cell carcinoma. In: Immunotherapy of Renal Cell Carcinoma. Marcel Dekker, New York.

Glenn G, Linehan, WM Hosoe S, Latif F, Yao M, Choyke P, Gorin M, Chew W, Oldfield E, Manolatos C, Orcutt ML, Wather M, Weiss G, Tory K, Jenmsson O, Lerman MI, Zbar B. Screening for von Hippel-Lindau disease by DNA polymorphism analysis. JAMA 1992;267:1226-1231.

Tory K, Latif F, Modi WQ, Schmidt L, Wei MH, Li H, Cobler P, Orcutt ML, Delisio J, Geil L, Zbar B, Lerman MI. A genetic linkage map of 96 loci on the short arm of human chromosome 3. Genomics 1992;13:275-286.

Latif F, Fivash M, Glenn GM, Tory K, Orcutt ML, Hampsch K, Delisio J, Lerman M, Beckett M, Cowan J, Weichselbaum R. Chromosome 3p deletions in head and neck carcinomas; statistical ascertainment of allelic loss. Can. Res. 1992;52:1451-1456.

Latif F, Tory K, Modi W, Graciano W, Douglas J, Rabbits P, Zbar B, Lerman MI. Molecular characterization of a large homozygous deletion in the small cell lung cancer line U2020: A strategy for cloning the putative tumor suppressor gene. Genes, Chromosomes & Cancer 1992;5:1-9.

Latif F, Modi WS, Duh F, Schmidt L, Li H, Geil L, Orcutt ML, Parton AH, Rabbitts, PH, Linehan M, Zbar B, Lerman M. Molecular and genetic characterization and physical mapping of eleven new markers detecting high frequency multiallele RFLPs on the short arm of human chromosome 3. Human Genetics, in press, 1992.

Li FP, Decker HJH, Zbar B, Stanton VP, Kovacs G, Seizinger BR, Aburantani H, Sanberg AA, Berg S, Hosoe S, Brown RS. Clinical and genetic studies of renal cell carcinomas in a family with a constitutional chromosome 3;8 translocation. Annals of Internal Medicine, in press.

Zbar B. Introduction to the biology and genetics of hereditary cancers. Seminars in Oncology Nursing, in press.

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

PROJECT NUMBER
Z01 CB 08578-03

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Preparation of a high resolution genetic map of human chromosome 3

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

Co-PI: B. Zbar and M.I. Lerman

Other: K. Tory PRI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Immunobiology

SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI-FCRDC, Frederick, MD 21702

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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

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

A genetic map has been constructed for the short arm of chromosome 3. The length of the sex-averaged 3p map is 114 cM. The length of the sex averaged 3q map is 123 cM. One hundred and twenty two loci were placed on the short arm of chromosome 3. Forty loci were placed on the long arm of chromosome 3.

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1991 through September 30, 1992

Research in the Laboratory of Mathematical Biology (LMMB) covers a broad range of theoretical and experimental studies of biological systems. These studies include molecular modelling, theoretical molecular calculations, membrane structure and function, immunology, pharmacokinetics, and physiological modelling studies. Basic understanding of these biological systems, serves as 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 that is utilized by researchers in the biomedical community at large. Many of the theoretical studies are possible only using the supercomputing facilities at the Biomedical Supercomputing Center, FCRDC.

Office of the Chief

Sequence Analyses in Molecular, Viral, and Cell 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 single point mutations. Searches for general trends involve comparisons of structures of related genes. General patterns are discerned in sequences fulfilling analogous functions, such as promoters, taken from a variety of genes/organisms or by searches for overall sequence characteristics such as those required by genome packaging (Le, Currey, Konopka, Nussinov, Maizel and Owens).

Supercomputing facilities were successfully upgraded to include a Cray YMP with 8 processors and 128 MW (>10^9 bytes) of real memory, a "on-line" mass storage tape facility, Convex minisupercomputer to manage mass storage, massively parallel sequence matching computer (MasPar 8000 processors), Vax 6620 front-end computers, and other peripherals. This is the only such supercomputing facility dedicated to biomedical research.

New analytical tools for studies of proteins and nucleic acids have been developed and implemented (Barber, Le, Maizel, Nussinov, and Owens). Numerical methods aid in the prediction of secondary structures, splice sites, promoters, and recombination sites in nucleic acids. Graphic representations reveal homology, and reverse complementarity. These programs were developed and have been installed on a variety of computer systems at

the Biomedical Supercomputing Center (BSC). RNA structures up to 9433 bases in size have been predicted. Methods to assess the significance of predictions have used Monte Carlo simulations, evolutionary comparisons and biochemical data. New sequences were compared with computerized databases to detect relationships with known proteins.

RNA secondary structure methods have been refined to include alternate energy parameters, extended Monte Carlo simulations, and comparative studies to establish firmly the uncommon structural features in subregions of a number of sequences of HIV and other retroviruses (Le, Nussinov, Currey, Shapiro, and Maizel). These predicted structural features have been correlated with biological features, leading to deeper understanding of the replication and expression processes in this group of viruses. In HIV-1 and related viruses, predicted stable features have been correlated with sites of tat-regulation elements, rev responsive elements and sites of translational frame-shift. Conserved secondary structure is predicted to be absent in regions of hypervariability in the envelope gene m-RNA's. Use of the supercomputer has allowed development of a look-up procedure for predicting stability of random sequences, which accelerates surveys nearly 100-fold. Monte Carlo techniques are being developed that yield greater than 80% correct prediction of t-RNA structures, for more than 100 examined sequences. Theoretical folding of the entire genome of HIV-1 on the Cray YMP 8/128 gives a global structure that retains all of the previously noted local structures.

Information Theory in Molecular Biology. Information theory, invented in the 1940's by Claude Shannon to describe the transmission of information across communication channels, is being used to understand molecular sequence patterns in genetic control systems (Schneider). The first results showed that most binding sites contain just as much information as is 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, called 'sequence logos' was invented which helps one to visualize the patterns at binding sites. The technique is superior to the well known consensus sequences. The sequenced logos are now being used to study the fine structure of binding sites. The fundamental processes of transcriptional control, translation, DNA replication and partition of DNA to daughter cells are actively being studied by using these techniques. Experimental work is also in progress on each of these to further test and strengthen 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 bio-molecules are able to do highly precise things. For example, chemical 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 new theory explains this as a coding similar to the error correcting codes used in telecommunications.

A further extension of the theory revealed how the Second Law of Thermodynamics constrains the number of things a bio-molecule can do with a given amount of energy dissipation. The limits on the famous Maxwell's Demon are now easily recognized as the channel capacity and the Second Law. These results set bounds which should aid in the design of molecular devices.

Molecular Biology of Glycosyltransferases. We continued our studies on structure and function of glycosyltransferases and of alpha-lactalbumin. These proteins determine the structure of the sugar chain of glycoproteins and glycolipids. Present studies are directed to probe into the interactions of complex carbohydrate structures with proteins and their influence on the cellular recognition processes. cDNA cloning results have shown that the Golgi glycosyltransferases have inverted membrane topology consisting of a short amino-terminal cytoplasmic tail, a hydrophobic-anchor domain, and the carboxyl-terminal portion of the protein that carries the catalytic activity.

By using deletion analysis and site directed mutagenesis of the region we are investigating the biological role of amino-terminal short cytoplasmic and membrane anchoring domains of this class of enzymes in mammalian cells. By exchanging the topological domains of glycosyltransferases we have identified membrane anchoring sequences of beta-1-4- and alpha-1-3-galactosyltransferases, and of alpha-2-6sialyltransferase, and have shown that they are essential for the synthesis and stability of these enzymes in mammalian system (Masibay, Qasba). The residues that are involved in anchoring these enzymes to Golgi membrane are being identified. To analyze the sugar donor/acceptor binding domains of the protein enzymatically active beta-1-4galactosyltransferase has been produced in E.coli (Boeggeman, Balaji and Qasba). Using pGEX vectors for the expression of the protein in E. coli either the full length or the amino-terminal deleted bovine galactosyl-transferase is expressed as a fusion protein, connected at the COOH-terminus of glutathione S-transferase. Deletion analysis show that the first 130 residues of the protein are not required for the enzymatic activity of the protein. Site directed mutagenesis has identified Cys 134 as an essential residue for the enzymatic function. Large quantities of these proteins are being purified for the X-ray crystal structure analysis.

Computer modelling methods are being used (Balaji, Rao and Qasba) to address the question "why does not alpha-lactalbumin bind any sugar while as its homologous protein c-type lysozyme with which it has both sequence and structural homology does bind and hydrolyse oligosaccharides"? The three dimensional structure of alpha-lactalbumin and lysozyme are very similar but the two proteins have different functions. With these modelling methods the amino acid residues in alpha-lactalbumin that block the entry of mono-saccharides into the site equivalent to the one identified as sugar binding site in the lysozyme are being identified. The side chain alterations in the protein that will allow binding of the sugar are being predicted and tested by the site directed mutagenesis of alpha-lactalbumin.

Molecular Structure. In the laboratory we are studying the 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. The direction of most of these studies is toward developing methods to facilitate the study of larger molecular assemblies.

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 and to evaluate them with simple residue-residue interactions. With a similar approach, we have been studying tertiary folding of RNA. 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, Jernigan) and should lead to new methods of drug design.

Molecular modeling has been proceeding in four areas: membrane proteins (Guy, Durell and Raghunathan), small peptides (Jiang and Jernigan), DNA helices (Jernigan, Zhurkin and Raghunathan), and DNA-protein interactions (Zhurkin). For the membrane proteins this model construction proceeds by combining experimental data with calculations of preferred locations and orientations of helices with respect to membrane boundaries, helix-helix packing, formation of charge pairs and disulfide bonds. Conformational models have been developed for the antibiotic magainin and cecropins; these models have improved our understanding of how they lyse cells and form channels. Models have been developed for three groups of channel proteins (voltage-gated potassium channels, annexins, and paradaxin), using a new structural motif for ion channels in which a β barrel is surrounded by α helices. Models were also developed for ways in which influenza virus hemagglutinins interact with each other and the viral and cellular membranes to cause membrane fusion. These models are based in part on the crystal structure of hemagglutinin.

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. One unresolved problem is what is the role of conformation in gene regulation; for DNA-protein interactions, these flexibilities and their asymmetries appear to play important roles. Models of other DNA forms, three-stranded helices and alternative base pairs are also being modelled and their function in recombination is being investigated.

Simulation, Analysis and Modelling of Physiological Systems, the oldest section of the laboratory continued development on the simulation, analysis, and modeling (SAAM/CONSAM) computer programs (Zech, Greif). The continued development of a version of SAAM30 which executes under the DOS operating system on personal computers and makes use of the Intel 80386 and 80486

processors continues by adding new software to the DOS versions which implements a help files, and graph legends.

CONSAM (the interactive environment for SAAM) graphics has been enhanced so that Legends can be posted on any plot in addition to labels. The capability to create a graphic element which can be completely independent of a graph name was added to the CONSAM graphics. Evaluation of the software to build a Microsoft Windows version of SAAM and CONSAM began by evaluating several new Fortran and C compilers which will produce executable code for use with the DOS Protected Mode Interface (DPMI) environment.

The SAAM project participated in the Fourth Mathematical Models in Nutrition Conference: Trace Element & Mineral Metabolism during development, held at Georgetown University in conjunction Dr. Meryl Wastney. We organized and conducted a compartmental modeling workshop starting the day before and running throughout this major meeting. In conjunction with the American Institute of Nutrition and the American Physiologic Society we presented SAAM and CONSAM at a 1992 FASEB symposium and participated in an associated computer demonstration area where approximately 400 researchers came to ask questions about SAAM and CONSAM.

The SAAM project also carried out collaborative research efforts (Zech), involving a large number of national and international investigators, in the analysis of data in the fields of lipid and lipoprotein metabolism, the testing of hypotheses concerning the quantitative description of the whole body metabolism and pharmacokinetics of cancer preventive selenium compounds, and the quantitative description of the metabolically significant vitamin A dynamics underlying homeostatic mechanisms that function to regulate the general physiological functions of growth and differentiation, reproduction, and vision and their relationships in cancer prevention. This effort also includes clinical duties and responsibilities for more than 37 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI. The SAAM project also carried out collaborative efforts, involving a large number of national and international investigators, in the chemical, pharmacological aspects of cancer chemotherapeutic and other drugs.

Image Processing

Analytic methods are continuing to be developed for the GELLAB-II software system with concentration on increasing: quantitation accuracy, automatic spot pairing, graphical user interface and integration of X-window based user graphics. A constant goal during this phase has been to simplify the user's role in performing the exploratory data analysis of a composite 2D gel database. Additional software was written toward achieving this goal. A major part of the effort has been in integrating the UNIX based GELLAB-II software with UNIX workstation based X-windows interactive graphics for portability. We have prepared a CRADA agreement to commercialize GELLAB-II which will result in its much wider use in an inexpensive platform and much better user support than we can provide for large numbers of users. We have continued our work on remote collaborative multimedia image-conferencing that allows GELLAB-II as well as a broad range of images from other fields and software to be shared over national computer networks.

Active Collaborative work has continued with the groups of Dr. J. Myrick (CDC/Atlanta), Dr. P. Rogan (Penn State Med. Sch.), Dr. R. Levenson (Duke Med. Sch.), Dr. P. Sonderegger(Zurich); Dr. T. Krekling (Univ. Norway), Dr. A. Grimshaw (Univ.VA); C. McGrath(AVD/FCRDC). Additional work was required to refine the exported version of GELLAB and to insure smooth updates of exported versions of the software.

Work has begun on exploring a relatively new class of algorithms, "genetic" algorithms, to facilitate the modeling of molecular structures, specifically RNA molecules. These algorithms have been shown in several applications to be parallelizable and rapidly convergent to solutions in a large search space consisting of many possible suboptimal results. Thus, our goal in this research has been to investigate representations that are appropriate for such "genetic" algorithms so that they may be applied to the problem of predicting RNA secondary and tertiary structure. The algorithms are being developed with massively parallel computing architectures in mind, including the MasPar (Shapiro, Navetta).

It is partially as a result of this research that much effort over the past year has been spent in investigating the various forms of massively parallel computer architectures to determine which, at this point in time, offers the best performance and computational environments. This has resulted in the acquisition of an 8000 processor MasPar computer system. The first phase of the genetic algorithm mentioned above is currently running on this system (Shapiro, Maizel, and Navetta). In addition, we are doing comparative studies with a newly developed sequence matching program based on the Smith/Waterman algorithm that runs on the MasPar. This algorithm is capable of performing 50,000,000 comparisons per second on a 4000 processor system (with Navetta and Maizel). The results from these runs are being compared with results utilizing other algorithms including another new algorithm running on a systolic array (Shapiro, Hoang and Schultz).

The genetic algorithm and the MasPar architecture have been integrated into the heterogeneous system that is being developed for RNA structure analysis. The system includes the facilities for activating algorithms that may run on various computer architectures that are accessible over a computer network. The RNA structure analysis system has also expanded to include more functionality for analyzing RNA conformations from various perspectives. This has involved the further enhancement of algorithms to explore secondary and tertiary structural motifs (Shapiro, Kasprzak).

The termination structures of lambda TR2 have been extensively studied (with Chang-Cheng, Lynch, Leason, Court, Friedman) and the 5' non-coding regions of Polio-virus and its mutational relationships to RNA secondary and tertiary structures are being characterized in regard to its functionality (Shapiro, Currey). The system has been used to study the fine structural details of the HIV-1 rev responsive element (RRE) (with Dayton, Konings, Powell, Butini, Maizel) and is currently being used to study the dimerization structure of HIV-1 (Shapiro, Baldwin).

Theoretical Immunology

The long-term aim of the Section is to understand the physiology and pharmacology of biological ligands to aid in the rational design of next-generation molecules for treatment of cancer and AIDS. The Theoretical Immunology Section (Weinstein) has focused on monoclonal antibodies, partly for their intrinsic biomedical interest and partly because they provide an important case study in the generic properties of biological ligands. Recent work has centered on quantitative modeling of the pharmacology of monoclonal antibodies. A computer program package called PERC was developed to integrate several hierarchical levels of information: 1) the whole-body and regional pharmacokinetics are modeled using the SAAM programs; 2) the microvascular transport properties are cast in terms of the non-equilibrium thermodynamics of coupled solute and volume flows; 3) percolation through tumor interstitium is expressed in the partial differential equations for convection- diffusion-reaction; 4) cellular binding and metabolism are represented as saturable compartments and sinks, respectively. The most interesting prediction from this work is that percolation of antibodies into tumors is retarded by the very fact of their successful binding to target antigens. This "binding site barrier" implies a possible role for antibodies of less than the highest possible binding affinity. A second program package (PERC-RAD) predicts the spatial and temporal distributions of radiation dose for antibody-radionuclide conjugates. PER-RAD indicates that the microscopic inhomogeneity of radiation dose for beta-emitting isotopes may be greater than previously thought. More generally, Dr. Weinstein and his colleagues hypothesize that the "binding site barrier" plays a major role in the microscopic pharmacology of other biological ligands, such as the lymphokines and cytokines, whether exogenous or endogenous. It may condition the evolutionary design of autocrine-paracrine molecules and affect the range of penetration for products of transfected cells injected in vivo. Experiments in an animal model have been completed, both for S.C. tumors and for micrometastases. They provide the first direct validation of the "binding site barrier" hypothesis.

In 1988 a serendipitous finding led members of the Section to identify a new molecular target for combination chemotherapy of HIV infection. Dipyridamole (Persantin) (DPM) is widely used as an oral agent for cardiovascular indications, and its best-defined mechanism of action is a potent inhibition of nucleoside transport into and out of cells. S. Szebeni and J.N. Weinstein found that DPM potentiates the activity of AZT and other dideoxynucleosides against HIV in monocytes and stimulated T-lymphocytes. Since DPM does not appear to potentiate the cytotoxic effect of AZT on human bone marrow progenitor cells in vitro, these findings suggested that DPM might increase the therapeutic index of AZT, and perhaps other dideoxynucleoside agents, in vivo. Even more surprisingly, in a T-lymphoblastoid cell line, DPM simultaneously potentiates the antiviral potency of AZT and decreases AZT's toxic effect on the cells by an order of magnitude. This dissociation of beneficial and toxic mechanisms of action leads to a large increase in the therapeutic index. The AZT-DPM combination has been approved for study within the AIDS Clinical Trials Group. Initial clinical trials of the AZT/DPM combination (in collaboration with groups at two other institutions) have been completed, and a trial with surrogate marker efficacy endpoints is being planned.

Aspects of this combination chemotherapy being studied within the section include: 1) the antiviral efficacy and cell toxicity; 2) the mechanisms of DPM activity; 3) molecular structure of the nucleoside transporter's binding site (by 3D-QSAR techniques); and 4) general analysis of combination therapy in cancer and AIDS. Because no published algorithm or computer package was adequate for analysis of data on the antiviral effect of drug combinations, a new approach was developed within the Section. It combines enzymology-based "pseudo-molecular" models with new statistical techniques. The resulting prototype computer program (COMBO) and concepts of interaction are proving useful for analysis of combination therapy in both cancer and AIDS.

During the last year, a combination of neural networks, expert systems methods, and classical statistics has been applied to various parts of the NCI cancer drug development process. The first result was a neural network capable of predicting a drug's mechanism of action from patterns of response in the 60-cell line NCI Cancer Drug Screen. An "Anti-Cancer Agent Predictions" Working Group (ACAP) has been formed to exploit aspects of artificial intelligence and information technology to help streamline the process.

Membrane Structure and Function

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

The first step of entry of enveloped animal viruses into cells is by fusion of the membrane of the virus with that of the target cell. This fusion process is catalyzed by viral envelope proteins. We have developed biophysical techniques to study the initial steps of viral envelope protein mediated membrane fusion. We label intact virus or cells with fluorescent dyes and observe the redistribution of those dyes during the fusion process. We study initial steps of HIV envelope protein-mediated membrane fusion, by continuous monitoring of fluorescent dyes during fusion using fluorescence spectroscopy and low light, image enhanced videomicroscopy. In particular we use HIV envelope protein expressed in cells by means of recombinant vaccinia virus and target membranes of defined composition with and without CD4 receptors. In this way we monitor fusion between cells or syncytium formation. The combination of studies employing HIV-expressing effector cells and defined target membranes facilitates the testing of hypotheses regarding the role of different factors in adhesion and fusion. Transmission of retrovirus between cells is thought to be associated with cell membrane fusion. In this way the virus is not exposed to the extracellular space and thereby hidden from the immune response. Thus, membrane fusion is a key element in the pathology of HIV, and an understanding of the mechanism of viral fusion might lead to the development of anti-viral therapeutic agents.

Structural Biology

The Structural Biology Section investigates the structure, topology, and topochemistry of cellular components. The emphasis is on the application of a unique system of methods developed in our laboratory. These methods combine modifications of freeze-fracture (label-fracture, replica-staining, fracture-label, fracture-flip, fracture-permeation, simulcast) with immunogold labelling procedures. Our new methods are being used to address many diverse problems in biology. At present these include junctional biology, oncogene biology, molecular virology, molecular neurocytology and parasitology, bacterial pathology and basic cell biology. Research projects involve collaboration with intramural scientists and joint efforts with scientists abroad.

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

PROJECT NUMBER
Z01 CB 08300-20 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
SAAM, Development and Applications for Analogic Systems Realization

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

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

Other Professional Personnel:
Peter C. Greif, M.D. — 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; Drs. H. Bryan Brewer, Daniel Rader, Alexander Mann, (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 ☐ (b) Human tissues ☒x(c) Neither
☐ (a1) Minors
☐ (a2) Interviews B

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

Continuing the development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems and its interactive version, Conversational SAAM or CONSAM, we have added software to the DOS versions which implements a help files and legends. CONSAM graphics has been enhanced so that Legends can be posted on any plot in addition to labels. Because labels are specific to each graph they are destroyed when the graph is deleted from the screen. The capability to create a graphic element which can be completely independent of a graph name was added to the CONSAM graphics. This new feature is called the LEGEND. Legends have the property that symbols and labels are right and left justified respectively from a midline partition. Legends can be rotated both clockwise and counter-clockwise in 90 and 180 degree increments so that they will always be parallel to an axis. All the symbols, line types, colors and sizes are available for each legend.

In comparison to normals, two features of apoB metabolism in patients with Familial hypercholesterolemia, the major secretory pathway through IDL and the absence of a catabolic loss of apoB from VLDL/IDL, greatly facilitate measuring the metabolic channeling of apoB into LDL. The data demonstrates a shift in the secretion of apoB from cholesterol ester-enriched to triglyceride-enriched particles in response to high carbohydrate, low fat feedings in normal subjects and patients with homozygous or heterozygous FH. In FH heterozygotes, however, the primary mechanism accounting for the reduction of LDL-cholesterol levels in response to high carbohydrate, low fat feedings was an increased clearance of LDL, presumably by the liver, which may not depend entirely on the integrity of the classical LDL (apoB,E) receptor pathway. This is significant because this pathway remains subject to enhancement by drugs and subjects with defective apoB,E-receptor can be treated.

Cooperating Units (Continued):

Juergan Schaefer, Molecular Diseases Branch, NIHLB; Richard E. Gregg, Squibb Inst. for Med. Res., Princeton, NJ; Drs. Phil Taylor, and Christine Swanson Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Res. Center, USDA; Dr. Blossom Patterson, Operations Res. Branch; Dr. Kevin C. Lewis, Human Nutrition Laboratory, NCI; Drs. Ba-Bie Teng and Allen Sniderman, Royal Victoria Hospital, Montreal, Quebec; Dr. Ahmed Kissebah, Univ. of WI; Dr. Andre J. Jackson, FDA, Rockville MD; Dr. Mary Mckenna, Univ. of MD Medical School, Baltimore Maryland.

PROJECT DESCRIPTION

Project #1. The development of mathematical and computer tools for the simulation of and analysis of bio-kinetic data and the implementation of these tools within the framework of SAAM and CONSAM.

Major Findings:

(1) SAAM and CONSAM Development: CONSAM for the 80386/DOS has been extended with three new features, On-line help, Graph Legends, and Macros.

On-line help: extended to include a HELP command displays the manual pages for more than 40 of the possible 200 CONSAM commands. These manual pages are present in the \SAAM\DICT (for dictionary) subdirectory in a standard text format and can be edited using most text editors. This approach has a drawback in that one is restricted to names which satisfy the rules for naming DOS files (i.e. the symbol y- will be found in a file called ynot and * will be found in a file called star, etc.). If the SWCON version of CONSAM is used, a copy of the file browser LIST is activated which allows complete and free movement within the entire set of manual pages. Using this browser all manual pages can be searched for selected key words.

Graphics: The PLOT command has been extended so that Legends can be included in a plot in addition to labels. Since labels are specific to each graph they are destroyed when the graph is deleted from the screen. Because of this fact, the capability to create a graphic element which can be completely independent of a graph name was added to the CONSAM graphics. This new feature is called the LEGEND. The description for the contents of each legend is stored in a file named SAAMLEG.DAT. Any particular legend can be posted to the screen. Each time a PLOT command is issued from CONSAM, a LEGEND is automatically posted which has the same legend name as the graph name associated with the plot command. Legends have the property that symbols and labels are right and left justified respectively from a midline partition. Legends can be rotated both clockwise and counter-clockwise in 90 and 180 degrees increments so that they will always be parallel to an axis.

Initial evaluation of the software to build a Microsoft Windows version of the system was begun by evaluating several new fortran and C compilers which will produce executable code for this DOS Protected Mode Interface environment.

(2) SAAM Workshops, Distribution, & Newsletter: In the past year we have been involved in several workshops. We participated in the Fourth Mathematical Models in Nutrition conference: Trace Element & Mineral Metabolism During Development, held at Georgetown University in conjunction Dr. Meryl Wasney. We organized and conducted a compartmental modeling workshop starting the day before and running throughout this major meeting. In conjunction with the American Institute of Nutrition and the American Physiologic Society we presented SAAM and CONSAM at a 1992 FASEB symposium and an associated computer demonstration area where approximately 400 researchers came to ask questions about SAAM and CONSAM.

Almost 90 copies of the SAAM/CONSAM software have been provided to the scientific community over the past 12 months in an effort to establish other centers in the collaborative effort. This involves combining and confronting theorist and experimentalist with topics which can profit from the application of computer simulation and computation and further serves to obtain the best experimental data for analysis an inclusion in data bases, such as the lipoprotein and selenium data bases. To this end we have extended the plan to continue to distribute these programs as necessary in the scientific community. SAAM, CONSAM, and the utility programs contained in this DOS disk set have been made available over the INTERNET network via the FTP (File Transfer Program) utility or over the public telephone network via kermit using a 9600 baud modem. A complete copy of the disks or updates are available.

Project Description #2:

Project #2 Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data. So that the Bio-Kinetic data collected will be applicable to compartmental analysis, this effort includes chairmanship of the Radioactive Research Drug Committee where all tracer studies come under review for scientific merit. This effort also includes 800 hours of patient contact and primary responsibility for 37 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

Major Findings:

(1) In collaboration with Dr. Fisher and Dr. Stacpoole: In 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.

Familial hypercholesterolemia (FH) is a common disorder in which lipoprotein cholesterol delivery to cells is impaired due to dysfunction of the apolipoprotein B,E receptor. The metabolic consequences of this abnormal state have previously been examined in vivo with the use of radio-iodinated apoB tracers, and repeated studies have demonstrated reduced clearance of LDL-apoB and presumably the LDL itself. Studies have also quantitated the importance of the apoB,E receptor pathway and identified alternate non-receptor-mediated routes for LDL catabolism. A protein labeled exogenously with radioiodine traces catabolic events but cannot be used to directly examine apolipoprotein biosynthesis and secretion. In contrast, an endogenous tracer, such as 3Hleucine, permits evaluation of these latter processes. We now report the results of such an investigation of the pathways of apolipoprotein B (apoB) secretion and its metabolic channeling in subjects heterozygous for FH and compare these to control studies performed in normal subjects.

The kinetics of apolipoprotein B (apoB) were measured in seven studies in heterozygous, familial hypercholesterolemic subjects (FH) and in five studies in normal subjects using in vivo tracer kinetic methodology with a 3Hleucine tracer. Very low density (VLDL) and low density lipoproteins (LDL) were isolated ultracentrifugally and LDL was fractionated into high and low molecular weight subspecies. ApoB was isolated, its specific radioactivity was measured, and the kinetic data were analyzed by compartmental modeling using the SAAM and CONSAM computer program. The pathways of apoB metabolism differ in FH and normal subjects in two major respects. Normals secrete >90% of apoB as VLDL, while one-third of apoB is secreted as intermediate density lipoprotein IDL/LDL in FH. Normals lose 40-50% of apoB from plasma as VLDL/IDL, while FH subjects lose none, metabolizing all of apoB to LDL. In FH, there is also the known prolongation of LDL residence time. The leucine tracer, biosynthetically incorporated into plasma apoB, permits distinguishing the separate pathways by which the metabolism of apoB is channeled. ApoB synthesis and secretion require 1.3 h. ApoB is secreted by three routes: 1) as large VLDL where it is metabolized by a dilapidation chain through a series of enzymatic reactions and exchanges; 2) as a rapidly metabolized VLDL fraction converted to LDL; and 3) and as IDL or LDL.

ApoB is metabolized along two pathways. The dilapidation chain processes large VLDL to small VLDL, to IDL, and subsequently LDL. The IDL pathway channels nascent rapidly metabolized VLDL and IDL particles into LDL. It thus provides a fast pathway for the entrance of apoB tracer into LDL, while the dilapidation pathway is a slower route for channeling apoB through VLDL into LDL. LDL apoB is derived in almost equal amounts from both pathways, which feed predominantly into large LDL. Small LDL is a product of large LDL, and the major loss of LDL-apoB is from small LDL.

In other words, two features of apoB metabolism in FH, the major secretory pathway through IDL and the absence of a catabolic loss of apoB from VLDL/IDL, greatly facilitate measuring the metabolic channeling of apoB into LDL. The question remains, how can this knowledge help treat patients with FH?

It has been previously noted that high carbohydrate, low fat diets have been known to lower serum total and low density lipoprotein (LDL) cholesterol in humans and to induce at least a transient elevation of circulating triglycerides. Changes have also been previously recorded in the lipid and apolipoprotein composition of lipoprotein particles, the magnitude of which appear to relate in part to the amount and type of carbohydrate in the diet and the underlying lipoprotein/apoprotein genotype of the individual.

Previously, the administration of a very high carbohydrate, low fat liquid diet of Vivonex in two homozygous receptor-negative FH patients with reduced circulating LDL cholesterol levels led in one patient to marked suppression of total body cholesterol synthesis. Other have observed in animal studies that high carbohydrate solid or liquid diets inhibited hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity and subsequent cholesterogenesis. To extend this investigation we examined and compared the cholesterol lowering response to high carbohydrate, low fat feedings in normal subjects and patients with homozygous or heterozygous FH, using the combined techniques of whole body cholesterol balance and endogenous tracer kinetics with tritiated leucine. Four normal, four heterozygous FH, and one FH homozygous subjects were studied on a basal (45% carbohydrate, 40% fat) diet and compared to studies undertaken during continuous nasogastric infusion of Vivonex (90% carbohydrate, 1% fat). For the entire group, the mean changes in total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides were - 90, 95, -14 mg/dl (all $P < 0.01$) and + 114 ($P < 0.02$) mg/dl, respectively.

Fecal sterol balance measurements demonstrated a 24% decrease in whole body cholesterol synthesis in normals, from 8.4 ± 4.4 (mean ± SD) to 6.4 ± 1.3 mg/kg per day and in FH subjects, a 58% decrease, from 11.4 ± 5.6 to 4.8 ± 1.7 mg/kg per day (both $P < 0.05$).

ApoB kinetic studies performed in two normals and three FH heterozygotes on both basal and Vivonex regimens, and the results were analyzed by compartmental modeling using SAAM and CONSAM computer program. Using these improved models to estimate apoB kinetics, total apoB production was not altered in a consistent manner by carbohydrate feeding. ApoB secretion, however, was shifted from the production of small VLDL/IDL-like particles to large VLDL by Vivonex, with an accompanying increase in intrahepatic assemblage time before secretion. In the two normal subjects, Vivonex induced an increase in apoB loss as VLDL/IDL; however, in the FH patients no such loss occurred. A decrease ($P < 0.05$) in the residence time of LDL-apoB occurred for all subjects and was the primary determinant of the fall in plasma LDL concentration, since LDL-apoB transport did not change consistently. Thus, in FH patients, a high carbohydrate, low fat diet results in suppression of cholesterol synthesis and a fall in plasma LDL concentration due to an increased plasma clearance rate for LDL.

The data demonstrates a shift in the secretion of apoB from cholesterol ester-enriched to triglyceride-enriched particles. In FH heterozygotes, however, the primary mechanism accounting for the reduction of LDL-cholesterol levels was an increased clearance of LDL, presumably by the liver, which may not depend

entirely on the integrity of the classical LDL (apoB,E) receptor pathway. This may ultimately prove to be the pathway subject to enhancement by drugs by which subjects with defective apoB,E-receptor can be treated.

(2) In collaboration with Dr. Daniel Rader and others in the Molecular Disease Branch of NHLBI: Apolipoprotein turnover studies were undertaken in 37 subjects in the last year. ApoA-IV was a major area of concentration.

Human apolipoprotein (apo) A-IV is a 46kD genetically polymorphic apolipoprotein in humans which is synthesized primarily in the small intestine. Though its precise function remains unclear, apoA-lV has been proposed to play a role in the metabolism of both triglyceride-rich lipoproteins (TRL) and high density lipoproteins (HDL). In a variety of experimental settings, apoA-lV has been shown to modulate lipoprotein lipase (LPL) activity, bind to endothelial cells, stimulate cholesterol afflux from adipose cells, activate lecithin:cholesterol acyltransferase (LCAT), participate in HDL particle conversion, and bind specifically to hepatic tissue. ApoA-lV has been found in dog peripheral lymph and in human mesenteric and thoracic duct lymph. As a result of its distribution and in vitro properties, apoA-lV is thought to play an important role in reverse cholesterol transport.

The major human ApoA-IV allele, known as apoA-lV-1, has an estimated gene frequency of 0.91, while the most common variant, apoA-lV-2, has a gene frequency of approximately 0.08. The molecular basis for this common polymorphism is a single G to T substitution in the apoA-lV-2 allele, resulting in the conversion of a glutamine to a histidine at residue 360 of the mature protein. This single amino acid substitution may have an important effect on plasma lipoprotein metabolism, and heterozygotes for apoA-lV-2/1 have been reported to have higher HDL cholesterol and lower triglyceride levels than apoA-lV-1/1 homozygotes. Others have reported that the apoA-lV-2 isoprotein has more alpha-helical structure, higher affinity for phospholipids, and greater ability to activate LCAT than the apoA-lV-1 isoprotein. These structural and functional differences suggest that apoA-IV-1 and apoA-lV-2 may have different in vivo metabolism as well.

Previous studies have been performed without regard to the apoA-lV phenotype of the source of the purified apoA-IV or the phenotype of the study subjects. Finally, the effect of apoA-lV phenotype on the metabolism of apoA-I, the major HDL apolipoprotein, is unknown. In order to gain further insight a series of investigations with the following objectives was undertaken:

1) to determine the kinetic parameters of the major isoprotein apoA-lV-1 in humans;
2) to directly compare the in vivo metabolism of the most common isoforms, apoA-lV-1 and apoA-lV-2; and
3) to determine the effect of these two major apoA-lV isoforms on apoA-I metabolism.

The in vivo kinetics of the major human apoA-lV isoproteins apoA-lV-1 and apoA-lV-2 were investigated in 13 normolipidemic human subjects. Purified apoA-lV-1 and apoA-lV-2 were radio-iodinated, reassociated with autologous plasma, and simultaneously reinfused into study subjects. Analysis of the kinetic data revealed a rapid mean fractional catabolic rate (FCR) for apoA-lV-1 of 2.61 +- 0.34 days-1 and for apoA-lV-2 of 2.28 l 0.32 days-1 (difference significant by paired t test, p = 0.003). The FCR of apoA-lV-2 in an apoA-lV-2/2 homozygote was slower at only 1.49 days-1 ($p<0.0001$). A significantly higher fraction of apoA-lV-2 than of apoA-lV-1 was found in association with high density lipoproteins (HDL), but the slower rate of apoA-lV-2 catabolism was related to delayed turnover of the non-HDL-associated fraction. The mean total production rate of apoA-lV was 15.9 mg/kg-d. Plasma apoA-lV concentration was related with production rate ($r=0.82$, $p=0.004$) and not related to catabolic rate ($r=0.06$). The metabolism of apoA-I, the major HDL apolipoprotein, was similar in an apoA-lV-1/1 subject, an apoA-lV-2/1 subject, and an apoA-lV-2/2 subject. We, conclude that: 1) apoA-lV is a rapidly turning over apolipoprotein in humans, with a transport rate, similar to that of apoA-I; 2) in contrast to apoA-I, plasma levels of apoA-lV are primarily determined by production rate in normolipidemic subjects; 3) the turnover of HDL-associated apoA-lV is markedly slower than that of non-HDL-associated apoA-lV; 4) the catabolic rate of apoA-lV-2 is slower than that of apoA-lV-1; and 5) the two major apoA-lV isoproteins do not have different effects on apoA-I metabolism.

(3) In collaboration with Dr. Blossom Patterson, Operations Research Branch, NCI; Drs. Phil Taylor and Christine Swanson, Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; we are investigating selenium kinetics. Little is currently known about the kinetics of selenium in humans. The Selenium Pharmacokinetics Study a joint study between NCI and USDA was designed to estimate basic pharmacokinetic parameters for two prototype forms of selenium: sodium selenite (inorganic) and selenomethionine (organic). Further, there was interest in whether these parameters would vary if either form was administered in a fasting or a non-fasting state as selenium is thought to be a cancer preventative in the proper dose and form. The metabolism of selenium is much more complex than was originally thought when the study was designed. This has led to the application of kinetic modeling to understand the details. The process of building a kinetic model for the metabolism of selenite and selenomethionine in humans is continued with the goal that when the models for the organic and inorganic forms have been completed they can be combined with the help of the computer to estimate the kinetics of a physiologic dose of selenium made up of both organic and inorganic forms.

The previously developed kinetic model for selenite metabolism was applied to a portion of the main study, the sixteen subjects were each given labeled selenite. Subjects were on a controlled diet for three days prior to dosing, and twelve days thereafter. This allowed us to determine their total intake of selenium, and helped assure that they would be in 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 these 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.

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.

A model for the analysis of the kinetics organic selenium, selenomethionine has been completed using the first six studies. This model will be used to compare the kinetics of organic selenium compounds and its modulation by fasting and 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 can not be examined simultaneously, this can only be accomplished with computer simulation.

Publications:

Roma P, Gregg RE, Bishop C, Ronan R, Zech LA, Meng MV, Glueck C, Vergani C, Brewer HB. Apolipoprotein A-I metabolism in subjects with a PST-1 restriction fragment length polymorphism and familial hypoalphalipoproteinemia. J Lipid Res 1990;31:1753-1760.

Zech LA, Greif PC, Raider DJ. Berman's simulation analysis and modeling. In: Malmendier CL, ed. Hypercholesterolemia, hypocholesterolemia, hypertriglyceridemia: Proceedings of the Fifth International Colloquium on Atherosclerosis in vivo kinetics. New York, New York: Plenum Press 1991;189-200.

Rader DJ, Castro G, Zech LA, Fruchart J, Brewer HB. Differential *in vivo* metabolism of high density lipoprotein particles LpA-I and LpA-I,A-II. J Lipid Res 1991;32:1849-1859.

Stacpoole PW, von Bergmann C, Kilgore LL, Zech LA, Fisher WR. Nutritional regulation of cholesterol synthesis and apolipoprotein B kinetics: Studies in patients with familial hypercholesterolemia and normal subjects treated with high carbohydrate diet, low fat diet. J Lipid Res 1991;32:1837-1848.

Swanson CM, Patterson BH, Levander OA, SA, Taylor PR, Veillon C, Zech LA. Human selenomethionine metabolism: A kinetic model. Human selenomethionine metabolism: Am J Clin Nutr 1991;54:917-926.

Fisher WR, Zech LA, Kilgore LL, Stacpoole PW. Metabolic pathways of apolipoprotein B in heterozygous familial hyperlipoprotenemia: Studies with a 3H-leucine tracer. J Lipid Res 1991;32:1823-1836.

Rader DJ, Gregg RE, Meng MS, Schaefer JR, Zech LA, Kindt MR, Benson MD, Brewer HB. In vivo metabolism of a mutant apolipoprotein, ApoA-I_{IOWA}, associated with hereditary systemic amyloidosis and low levels of HDL. J Lipid Res 1992;33, in press.

Patterson BH, Zech LA. Development of a model of selenite metabolism in Man. Am J Nutr, in press.

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

PROJECT NUMBER
Z01 CB 08303-20 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Membrane Fusion Mediated by Viral Spike Glycoproteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,
Robert Blumenthal, Ph.D., Chief, Membrane Structure & Function Sect., LMMB, NCI
Other Professional Personnel:

Anu Puri, Ph.D.	Visiting Associate	LMMB, NCI
Charles Pak, Ph.D.	IRTA	LMMB, NCI
Mathias Krumbiegel, Ph.D.	Visiting Fellow	LMMB, NCI
Dimiter Dimitrov, Ph.D.	Visiting Scientist	LMMB, NCI

COOPERATING UNITS (if any)
Dr. Hana Golding, CBER; Dr. Joel Lowy, AFFRI; Dr. Michel Ollivon, CNRS, France; Dr. Abraham Loyter, Hebrew University, Israel; Dr. Joshua Zimmerberg, NICHD; Drs Yi-der Chen and F. Booy, NIDDK; Drs E. Berger, B. Moss, C. Broder, (continued)

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Membrane Structure & 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 ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

B

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

The research goals in the Membrane Structure and Function Section are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. We are specifically studying the mode of action of the envelope protein of HIV, the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus. Specific topics include: i) development of fluorescent methods to study kinetics and extent of adhesion and fusion using intact and reconstituted virions, and liposomes and cells as targets; ii) development of methods to analyze reconstitution of viral spike glycoproteins; iii) functional reconstitution of viral spike glycoproteins into lipid vesicles iv) studies of mechanism based on an allosteric model: the role of ligand binding, conformational changes and cooperativity of viral spike glycoproteins in mediating membrane fusion v.) studies of the effects of modifications of viral spike glycoproteins by pH temperature, enzymes, and chemicals on their fusogenic activities vi) studies of the relationship between virus-induced membrane destabilization (permeability changes) and fusion vii.) Studies of viral entry into the cell by endocytosis using fluorescent techniques. viii.) Application of image processing using video-enhanced fluorescent microscopy controlled by a computer to analyze viral entry pathways. ix) Examination of the disposition of the fusion protein after the fusion event; x) Identification of possible fusion intermediates; xi) Development of methods to study fusion activity of mutants of viral proteins using cloned viral membrane protein sequences expressed in transfected cells; xii) Structural studies of viral proteins; xiii) Development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

Cooperating Units (continued):

R. Willey, M. Martin, NIAID; Dr S.J. Morris, UMKC, Kansas City; Dr. D.P. Sarkar, Univ. of New Delhi, India; S. Rottem and M. Barile, CBER.

PROJECT DESCRIPTION

Major Findings:

1. *Redistribution of viral lipid, protein and RNA upon influenza virus fusion.*

We examined by video microscopy the redistribution of viral lipid, protein and RNA upon incubation of influenza-erythrocyte complexes at low pH and different temperatures. Lipid was monitored using the fluorescent dye, R18, influenza hemagglutinin was labeled with FITC, and viral RNA was observe by staining with acridine orange. At temperatures >15°C and pH 4.9, lipid, protein and RNA redistributed at about the same rates. However, at temperatures below 15°C, low pH incubation for >8 min resulted in lipid redistribution, whereas the protein and RNA remained with the virus. These studies are consistent with the formation of fusion junctions which allow passage of lipid while the protein and RNA is still retained with the virus.

2. *Effects of dextran sulfate on influenza hemagglutinin-mediated fusion.*

We examined the mode of action of dextran sulfate as an inhibitor of viral fusion. The polymer neither affected binding of virus or of hemagglutinin-expressing cells with red blood cells, nor the low pH-induced conformational change, but it strongly inhibited low pH-induced fusion. The inhibitory effect occurred only if the polymer was added at early steps of the fusion reaction, and if it was continuously present during the fusion reaction.

3. *Effect of non-adsorbing polymers on viral fusion.*

Non-adsorbing polymers such as dextran and polyethyleneglycol (PEG) enhance binding as well as rates and extents of fusion of influenza virus with erythrocytes. The effects of the non-adsorbing polymers were in the concentration range from 0-10 wt%, far below the concentrations required to overcome hydration repulsion forces. The enhancing effects were dependent on molecular weight of non-adsorbing polymer, and only occurred at m.w.>1500. High molecular weight PEG induced fusion of vesicular stomatitis virus with intact erythrocytes, which do not serve as targets of VSV fusion in the absence of the polymer. The time delay between triggering and the onset of influenza virus fusion was significantly reduced in the presence of non-adsorbing polymers.

4. *Control of virus-cell fusion by host cell lipid composition.*

Virus-induced cell fusion has been examined in a series of stable cell lines which were originally selected for resistance to the fusogenic effects of PEG.

The PEG fusion-altered cell lines are characterized by their unusual lipid composition, including marked elevation of saturated fatty acids and the presence of an unusual ether-linked neutral lipid. For vesicular stomatitis virus (VSV) and influenza virus susceptibility to fusion was found not to be correlated with susceptibility to PEG-induced fusion. These data indicate that the virus finds its appropriate fusogenic domain on the target membrane.

5. *Fusion of plasma membrane vesicles (PMV) with VSV G protein expressing cells*

We have studied fusion between labeled PMV, prepared from Vero cells by hypotonic lysis and Vero cells expressing VSV G using an assay for lipid mixing based on the relief of self-quenching of octadecylrhodamine (R18) fluorescence. Fusion began immediately after lowering the pH below 6, and showed an approximately exponential time course. The pH dependence of PMV fusion paralleled that observed for VSV-cell fusion and VSV-induced syncytia formation. The assay opens the possibilty to study the fusion activity of mutant envelope proteins expressed in cells.

6. *Kinetics of low pH-induced conformational changes in influenza HA.*

Conformational changes result in increased hydrophobicity of the molecule as monitored by binding to the fluorescent dye, Nile Red, and in a change in tryptophan fluorescence. Fast and slow components of the conformational change are revealed which could be related to changes in the stem region and in the globular region, respectively. The temperature and pH dependent kinetics are related to the functional activity of the protein in promoting viral fusion.

7. *Reconstitution of paramyxo-viral envelopes.*

Viral envelopes containing the fusion protein (F) and hemagglutinin-neuraminidase (HN) (F,HN-virosomes), or F only (F-virosomes) were constructed by dissolution of the virus in detergent followed by purification of the envelope proteins, and removal of the detergent. To insure binding of F-virosomes to target cells we either provided an external ligand (WGA), or used Hep G2 cells, which contain the asialoglycoprotein receptor as a binding site for the F protein. Using a lipid mixing assay we show that the F-virosomes were just as efficient in fusing with target cells as F,HN-virosomes provided that an appropriate binding site was present.

Description of AIDS Research

8. *The role of Ca2+ in HIV-1-Env-mediated cell fusion.*

Calcium ions are required for fusion of a wide variety of artificial and biological membranes. To examine the role of calcium ions for cell fusion mediated by interactions between CD4 and the HIV-1 envelope glycoprotein (gp120-gp41), we used two experimental systems: i) cells, expressing gp120-gp41 and its receptor CD4, each encoded by recombinant vaccinia viruses, and ii) chronically infected cells, producing low levels of HIV-1. Fusion was

measured by counting the number of syncytia and by monitoring the redistribution of fluorescence dyes utilizing video microscopy. Syncytia did not form in solutions without calcium ions. Addition of calcium ions restored the formation of syncytia. EDTA and EGTA completely blocked syncytia formation in culture mediums containing calcium ions. Membrane fusion as monitored by fluorescence dye redistriubution also required calcium ions. Cell fusion increased with increasing calcium ion concentration from 100 μM to 10 mM, but was not affected by magnesium ions in the concentration range from 0 to 30 mM. Fibrinogen and fibronectin did not promote fusion in the absence of calcium ions. We conclude that calcium ions are essential for cell fusion mediated by the CD4-HIV-1 envelope glycoprotein interaction.

9. *Interactions of CD4 containing plasma membrane vesicles (CD4-PMV) with HIV-1 and HIV-1-Env protein-expressing cells.*

CD4-PMV were prepared by hypotonic lysis of HeLa cells expressing CD4 after infection with recombinant vaccinia virus containing the CD4 cDNA. The CD4-PMV carried up to 680 CD4 molecules per vesicle. Their fusion with cells, expressing gp120-gp41 after infection with recombinant vaccinia virus, was monitored by fluorescence video microscopy by using lipophilic fluorescent dyes. Fluorescence changes as a result of fusion occurred within 30 min at 37°C, and little fluorescence changes were seen with cells expressing the non-cleaved HIV-1 envelope glycoprotein (gp160). The preincubation of CD4-PMV with HIV-1 reduced its infectivity 10-fold. The CD4-PMV were more effective in inhibiting syncytia formation than sCD4. These results demonstrate that CD4-PMV could be used to study the mechanisms of HIV-1 envelope-mediated fusion and have the potential to inactivate HIV-1.

10. *Quantitation of HIV-1 infection kinetics.*

The infection of permissive T cells (CEM and H9) by HIV-1 (pNL4-3 and IIIB) in tissue cultures proceeded in three stages: i) no detectable virus production, ii) a sharp increase in virus concentration reaching a peak, and iii) a decline in virus production. The peak in virus concentration correlated with a peak in the cell aggregation, an increase in the number of dead cells and a decrease in the total number of cells. The delays in the peaks of infection were proportional to the logarithm of the multiplicities of infection (MOI). An empirical formula was found which describes the overall kinetics of HIV-1 accumulation in the cell culture supernatants at different MOI. This formula follows from a model of virus spreading by subsequent rounds of infection and allows quantitation of virus infectivity. The analysis of the model and the experimental data showed that the criticalparameter for the kinetics of HIV-1 infection is the infection rate constant k = lnn/ti, where n is the number of infectious virions produced by one cell (17 to 26), and ti is the time for one cycle of virus replication (3 days), and that the infectivity of HIV-1 during its spread in tissue cultures is 20 to 103 times higher than its infectivity in cell-free culture supernatants of infected cells. These results may provide new insight into mechanisms of AIDS progression, and quantitation of HIV-1 infectivity and transmission.

11. *Control of HIV-1-Env glycoprotein mediated cell fusion by the host cell membrane.*

The inability of animal cells expressing human CD4 to fuse with cells expressing the HIV-1 envelope glycoprotein was investigated using vaccinia vectors to express each protein. Murine or monkey cells expressing human CD4 did not form syncytia upon mixing with cells envelope glycoprotein, even when both molecules were expressed at extremely high surface levels. Using fluorescence video microscopy to monitor fluorescent dye transfer between fusing cells, the block was shown to occur at the level of fusion of individual pairs of cells. In order to gain insight into the basis for this fusion block, we tested the ability of probe cells expressing HIV-1 envelope glycoprotein to transfer fluorescent dye to CD4-expressing human/animal hybrid giant cells. The hybrid giant cells were generated either by CD4-gp120-gp41 mediated cell fusion, or by low pH induced fusion of vaccinia-infected cells. We observed that gp120-gp41 expressing probe cells efficiently fused with CD4 expressing human/animal hybrid giant cells, but not with giant cells derived by fusion of animal cells alone. Fusion was independent of whether the CD4 was originally expressed in the human or the animal cell. These results suggest that the block to fusion is not due to dominant inhibitory components in the animal cell; rather, they indicate that human cells contain additional component(s) which, when transferred to the CD4-bearing animal cells, confer the ability to undergo membrane fusion mediated by the HIV-1 envelope glycoprotein.

12. *The role of accessory molecules in entry of HIV-1 into cells.*

The role of the cell surface adhesion molecule, LFA-1, in HIV-1 envelope glycoprotein mediated cell fusion was examined by using LFA-1 negative EBV transformed cell lines from two leukocyte adhesion deficiency patients. These cells were infected with recombinant vaccinia virus to express the HIV-1 envelope glycoprotein and co-cultured with CD4-positive subclones of the human T cell line CEM, which express either normal or low levels of LFA-1. We found that the cell lines expressing low levels of LFA-1 formed much smaller and fewer syncytia compared to the clones expressing normal levels of LFA-1, but both clones fused equally well with the LFA-1-negative B cells expressing the HIV-1 envelope glycoprotein as monitored by a fluorescent dye redistribution assay. Furthermore, monoclonal antibodies against the LFA-1 molecules reduced the number of syncytia formed but had no effect on membrane fusion. Thse findings demonstrate that the adhesion molecule LFA-1 does not play a crucial role in the early events of HIV-1 envelope glycoprotein mediated cell membrane fusion, but rather contributes to the later events leading to giant cell formation.

13. *Fusion of HIV-1-Env protein-expressing T cells with B cells containg surface Ig receptors specific for haptenated gp120, or unmodified gp120.*

We developed a new model system, which utilizes the anti-TNP/TNP interactions between a panel of TNP-specific human B cell lines and TNP-haptenated HIV-1 envelope expressing T cells. This system allowed us to examine the possible contribution of antigen-specific immunoglobulin receptors on fusion mediated

by the HIV-1 envelope. CD4-negative T cells (12E1) were infected with recombinant vaccinia virus encoding the HIV-1 envelope and mixed with TNP-specific EBV-transformed B cell line (1:13), which expresses low levels of CD4. Few syncytia were formed between the gp120-gp41 expressing effector cells and the B cells. The TNP-haptenation of the effector cells, however, increased syncytia formation 10-fold. The formation of syncytia was inhibited by sCD4, OKT4A, anti-TNP antibody, and TNP-BSA. No enhancement of syncytia was observed if the T cells were first TNP-modified and then infected with gp120-gp41 vaccinia. We also showed that gp120 was TNP-haptenated by using ELISA. In addition, a CD4-negative TNP-specific B cell line formed syncytia with TNP-modified gp120-gp41 expressing cells. We conclude that in addition to CD4, certain B-cell-Ig receptors which bind to gp120 may induce conformational changes leading to cell membrane fusion and may contribute to the depletion of anti-gp120 B cells in HIV-1 infected individuals.

14. *Invasion of lymphocytes by mycoplasm.*

We investigated the ability of M. fermentans (strain incognitus) to fuse with lymphocytes by monitoring the dequenching of R18 which was incorporated into the mycoplasma cell membrane. Fusion of M. fermentans was detected with Molt-3 (CD4-positive) cells, 12E1 (CD4-negative) cells and primary peripheral blood lymphocytes, but fusion yields were low (5-12% of the mycoplasma cells fused). The rate of fusion was temperature dependent. Following a short lag period, fusion at 37°C was completed within 60 min. Fusion was inhibited by EDTA (10 mM), by anti-M. fermentans antisera and by pretreatment of the mycoplasma with proteolytic enzymes, suggesting that a surface-exposed proteinaceous component is involved in the fusion process.

15. *Role of CD4 structure in HIV-1-Env glycoprotein-mediated fusion.*

Using CD4/CD8 chimeric molecules stably expressed in target cells, we show that the proximal membrane domains of CD4 are important for the fusion kinetics of cells, expressing the HIV-1 envelope glycoprotein, but do not affect significantly the extent of fusion.

16. *Role of envelope glycoprotein surface density.*

The fusion of cells and formation of syncytia were dependent on the level of surface expression of the HIV-1 envelope glycoprotein. An increase in the surface density of gp120-gp41 led to an increase in both, the rate and extent of fusion.

Publications:

Blumenthal R, Puri A, Dimitrov DS. Initial steps of enveloped virus entry into animal cells. In: Verna R, Nishizuka Y, eds. Biotechnology of cell regulation. New York, New York:Raven Press 1991;115-133.

Blumenthal R, Schoch C, Puri A, Clague MJ. A dissection of steps leading to viral envelope protein-mediated membrane fusion. Ann NY Acad Sci 1991;635:285-296.

Cao CJ, Lockwich T, Scott TL, Blumenthal R, Shamoo AE. Uncoupling of Ca2+ transport from ATP hydrolysis activity of sarcoplasmic reticulum (Ca2+ + Mg2+)-ATPase. Mol Cell Biochem 1991;103:97-111.

Clague MJ, Knutson J, Blumenthal R, Herrmann A. The interaction of influenza hemagglutinin amino terminal peptide with phospholipid vesicles: A fluorescent study. Biochemistry 1991;30:5491-5497.

Dimitrov DS, Golding H, Blumenthal R. Initial stages of HIV-1 envelope glycoprotein mediated cell fusion monitored by a new assay based on redistribution of fluorescent dyes. AIDS Res Hum Retroviruses 1991;7:799-805.

Dimitrov DS. Membrane fusion kinetics. In: Chang DC, Chassy BM, Saunders JA, Sowers AE, eds. Guide to electroporation and electrofusion. Orlando, Florida:Academic Press, 1991;10:155-166.

Herrmann A, Zachowski A, Devaux PF, Blumenthal R. Control of fusion of biological membranes by phospholipid asymmetry. In: Ohki S, ed. Cell and model membrane interactions. New York, New York:Plenum Publishing Co 1991;89-113.

Kaplan D, Zimmerberg J, Puri A, Sarkar DP, Blumenthal R. Single cell fusion events induced by influenza hemagglutinin: Studies with rapid-flow, quantitative fluorescence microscopy. Exp Cell Res 1991;195:137-144.

Dimitrov DS, Blumenthal R. Kinetics of intermembrane interactions leading to fusion. In Ohki S, ed. Cell and model membrane interactions. New York, New York: Plenum Publishing Co 1992;229-247.

Dimitrov DS, Hillman K, Manischewitz J, Blumenthal R, Golding H. Kinetics of soluble CD4 binding to cells expressing human immunodeficiency virus type 1 envelope glycoprotein. J Virol 1992;66:132-138.

Guy HR, Durell SR, Schoch C, Blumenthal R. Analysing the fusion process of influenza hemagglutinin mutagenesis by mutagenesis and molecular modeling. Biophys J 1992;62:113-116.

Dimitrov DS, Willey R, Martin M, Blumenthal R. Kinetics of HIV-1 interactions with sCD4 and CD4+ cells. Implications for inhibition of virus infection and initial steps of virus entry into cells. Virology 1992;187:398-406.

Dimitrov DS, Hillman K, Manischewitz J, Blumenthal R, Golding H. Kinetics of interaction of sCD4 with cells expressing HIV-1 envelope and inhibition of syncytia formation. AIDS 1992;6:249-256.

Sato H, Ornstein J, Dimitrov DS, Martin M. Cell-to-cell spread of HIV-1 occurs within minutes and may not involve participation of virus particles. Virology 1992;186:712-724.

Clague MJ, Schoch C, Blumenthal R. Towards a dissection of the Influenza Hemagglutinin mediated membrane fusion pathway. In: Bentz J, ed. Viral fusion mechanisms. Boca Raton, Florida:CRC Press, in press.

Dimitrov DS, Franzoso G, Salman M, Blumenthal R, Tarshis M, Barile M, Rottem S. Mycoplasma Fermentans, Incognitus strain cells are able to fuse with T lymphocytes. FEBS lett, in press.

Golding H, Dimitrov DS, Blumenthal R. LFA-1 adhesion molecule is involved in HIV-1 env-mediated syncytia formation but not in the early stages of cell membrane fusion. AIDS Res Hum Retroviruses, in press.

Herrmann A, Clague MJ, Blumenthal R. The role of target membrane structure in fusion with influenza virus: effect of modulating erythrocyte transbilayer phospholipid distribution. Membrane Biochemistry, in press.

Morris SJ, Zimmerberg J, Sarkar DP, Blumenthal R. Kinetics of cell fusion mediated by viral envelope proteins. Methods Enzymol, in press.

Puri A, Dimitrov DS, Golding H, Blumenthal R. Interactions of CD4+ plasma membrane vesicles with HIV-1 and HIV-1 envelope glycoprotein expressing cells" J AIDS, in press.

Puri A, Clague MJ, Schoch C, Blumenthal R. Kinetics of fusion of envelope viruses with cells. Methods Enzymol, in press.

Walter A, Lesieur S, Blumenthal R, Ollivon M. Size characterization of Liposomes by HPLC. In: Gregoriadis G, ed. Liposome technology. 2nd edition, Boca Raton, Florida:CRC Press, in press.

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

PROJECT NUMBER
Z01 CB 08320-17 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Peptide Conformations and their Binding Sites

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

Robert Jernigan, Ph.D.	Deputy Chief, LMMB, NCI	
Other Professional Personnel:		
Shou-ping Jiang	Visiting Scientist	LMMB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI
Lynn Young, Ph.D.	Staff Fellow	LMMB, NCI

COOPERATING UNITS (if any)
Saul Jacchieri, Dept. of Chem., Univ. of FL; Dennis Torchia, NIDR; David Covell, BSC, PRI, FCRDC.

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0.0

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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 extremely large numbers of conformations accessible to peptides of size 10 to 20 residues, make them difficult to consider both theoretically and experimentally. Comprehensive sets of their conformations are nearly impossible to consider. A new approach to combine calculations with specific information has been developed. The constraining information could originate either from experiment or from a molecular model with specified interactions. This approach to consider random peptide conformations with constraints has been formulated in the context of helix-coil theory.

In other studies of the use of NMR data as constraints, ways to perform thorough conformational searches have been developed in order to view all conformations that are consistent with the experimental data. The number of such alternative forms provides a direct measure of the extent of structural under-determination.

Protein surfaces are being examined in order to develop methods to specify likely binding sites for peptides. This is the first stage in a new approach to inhibitor design to develop new therapeutic agents.

PROJECT DESCRIPTION

Major Findings:

In most molecular calculations on large structures, an initial model or structure is required, and calculations typically refine this starting structure. It is interesting to develop methods that can perform larger conformational searches based more directly on experimental data. An example of such data might be a salt bridge in an alpha helix which could be either postulated or based on experiment. We have developed a formalism for performing fairly broad calculations of molecular conformation with such limited information. This approach might permit a crude determination of the relative feasibilities of several alternative models at an earlier stage in the development of a molecular model. The aim has been to bridge the gap between non-quantitative molecular model building and more rigorous but less directed molecular calculations. An application was made to salt bridges in the C peptide of ribonuclease A.

Known structures with bound peptides have been studied intensively in order to develop ways to specify hot spots for peptide binding. This new approach is quite successful when compared to known structures. The result is a cluster of points immediately exterior to the protein. In a next stage, the best amino acids to place on these points will be determined.

Publication:

Jacchieri S, Jernigan RL. Variable ranges of interactions in polypeptide conformations with a method to complement molecular modeling. Biopolymers 1992, in press.

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

PROJECT NUMBER
Z01 CB 08341-14 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structure/Function Relationships in Molecules for Treatment of Cancer & AIDS

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

John N. Weinstein, M.D., Ph.D.	Chief, Theoretical Immunology Section	LMMB, NCI
Other Professional Personnel:		
Vellarkad Viswanadhan, Ph.D.	Visiting Scientist	LMMB, NCI
Hitoshi Sato, Ph.D.	Visiting Fellow	LMMB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Theoretical Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0.0

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☐ (a1) Minors
☐ (a2) Interviews B

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

To understand the molecules important to our projects on the pharmacology of biological ligands (Z01 CB 08366-09) and combination therapy (Z01 CB 08392-04), we have explored several innovative ways of analyzing protein structure-function relationships:

1. HAL, HALP, & HALCO. We developed these statistical mechanics-based algorithms to evaluate amphipathic helical structures and more general structural motifs in proteins and peptides, including those of the HIV-1 envelope.

2. A joint prediction algorithm. This new method predicts peptide secondary structure by concatenating the predictions of several different algorithms (e.g., based on neural networks, information theory, sequence homology, hydrophobicity, loop potential, and amphipathicity.) This initially *ad hoc* approach was formalized in an algorithm called Q7-JASEP (for Q7-based Joint Algorithm for Secondary Structure Prediction).

3. 3-dimensional quantitative structure-activity relationship (3D-QSAR) studies. 3D-QSAR analysis was done using a set of 20 nucleoside transport inhibitors. The nucleoside transport protein is a major target for dipyridamole, which we have found to potentiate the activity of AZT against HIV-1 (see project #Z01 CB 08392-04). Clinical trials of AZT/dipyridamole in HIV-infected patients are in progress, and the 3D-QSAR predictions can be used to direct the design of analogue inhibitors for study.

4. Thermodynamic cycle perturbation (TCP) on HIV-1 protease-inhibitor complexes. We are using TCP methods to "mutate" one peptidomimetic inhibitor of HIV-1 protease computationally into another and predict the binding free energy of the latter peptide from that of the former. Interest of this analysis arises from the fact that inhibitors of HIV-1 protease are among the most promising new agents for treatment of HIV infection. We are currently collaborating in the analysis of their behavior in combination with dideoxynucleosides (part of Z01 CB 08392-04).

PROJECT DESCRIPTION

Major Findings:

(1) The HAL (Helical Amphipathicity Locator) algorithm and computer program set for analysis of structure/function relationships in proteins was successful in predicting crystallographic helix from sequence information. It also provided information for cluster mapping of several categories of amphipathic and non-amphipathic sequences. The related packages HALP, HALPSTAT, COPHAL, and HALCO were developed to generalize the algorithm. HAL was used to analyze structural features of the HIV-1 envelope polyprotein, HLA allotypes, and serum apolipoproteins, *inter alia*.

(2) HAL was also used to predict suramin binding sites on basic fibroblast growth factor. These predictions were used by collaborators for rational design of next-generation suramin analogues. (Suramin is the most promising new drug for advanced prostate carcinoma, and we are collaborating in the analysis of its effect in combination with other antitumor drugs (as part of project #Z01 CB 08392-04).

(3) We formulated a new joint prediction approach to protein secondary structure as an "expert system" and used it to predict the structure of folate binding proteins (which are important in the cellular handling of folates and antitumor drugs such as methotrexate). The method was then improved and automated in an algorithm that we call Q7-JASEP (for Q7-based Joint Algorithm for Secondary Structure Prediction). Q7-JASEP combines predictions from neural networks, information theory, sequence homology, hydrophobicity, loop potential, and amphipathicity. By the criterion of correlation coefficient (Q7), Q7-JASEP is better at predicting secondary structure in alpha/beta proteins and in general globular protein databases than any of the individual component methods.

(4) 3D-QSAR. The most robust predictions for the nucleoside transport inhibitors (see project #Z01 CB 08392-04) are (i) that the nucleobase plays a larger role in binding than previously thought and (ii) that the 5'-OH group is also very important to the energetics of binding.

(5) Thermodynamic cycle perturbation studies of HIV-1 protease-inhibitor complexes. These CRAY-intensive calculations revealed a good agreement between predicted and empirical binding constants for a "mutated" peptidomimetic inhibitor. This is just a first test for the protease system, but, if supported by further studies, this method could be useful in predicting features for design of additional inhibitor molecules.

AIDS research:

As indicated on the HHS-6040 and in the Major Findings section:

HAL and its associated program sets have been used to predict structure/function relationships in the HIV-1 envelope. The initial emphasis was on T-cell epitopes.

The 3D-QSAR algorithm was applied to analysis of the binding site of the nucleoside transporter. The transporter, an intrinsic membrane protein, is the principal target for dipyridamole. We found that dipyridamole potentiates the activity of AZT and ddC against HIV-1 in culture and have proceeded to clinical trials in collaboration with two other institutions (see project #Z01 CB 08392-04). In this 3D-QSAR analysis, we examined the structures of 20 transport inhibitors and looked for correlation between particular structural features (e.g., a 5'- hydrophilic group such as -OH) and the binding constant for that inhibitor with the protein. The algorithm thus led to predicted features of the binding site and to predictions for use in designing better-binding drugs. These predictions have not yet been tested.

With respect to the TCP studies on HIV-1 protease, see (5) above.

Publications:

Viswanadhan VN, Denckla B, Weinstein JN. A joint prediction algorithm (Q7-JASEP) improves the prediction of protein secondary structure: Application to alpha/beta proteins. Biochemistry 1991;30:11164-11172.

Reddy MR, Viswanadhan VN, Weinstein JN. Relative differences in the binding free energies of HIV1 protease inhibitors: A thermodynamic cycle perturbation approach. Proc Natl Acad Sci USA 1991;88:10287-10291.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08363-10 LMMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Membrane Protein Modelling

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

H. Robert Guy, Ph.D. Senior Staff Fellow LMMB, NCI

Other Professional Personnel:

Stewart Durell, Ph.D. IRTA 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:
2.0	2.0	0.0

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The primary goals of this project are to develop methods to predict structures of membrane proteins from their sequences and available experimental data, to use these methods to develop structural models of specific membrane proteins, and to work with experimental groups to test these models. We have developed a hierarchical approach to modeling membrane proteins. In the first phase we predict which segments cross the membrane and the secondary structure of these segments, in the second phase we predict the relative positions and orientations of the transmembrane segments, and in the third phase we use computer graphics and molecular mechanics energy calculations to produce models that predict positions of all atoms in the regions of the proteins that are modeled. We have developed models through the third phase for members of the following families of proteins: delta lysin, magainins, cecropins, alamethicin, pardaxin, annexins, and voltage-gated potassium channels. Most of our time during the past year was spent developing Phase III models of voltage-gated potassium channels and models of the influenza virus fusion process. We have now established collaborations to begin the fourth phase of modeling of the potassium channel in which energetic factors such as water, lipids, ions, membrane voltages, and entropy are included in the energy calculations and the fifth phase in which functional properties such as gating, ion permeation, and drug binding mechanisms are modeled.

PROJECT DESCRIPTION

Major Findings:

We have continued our efforts to model the structure of voltage-gated sodium, calcium, and potassium channels. Our controversial prediction that the ion selective pore of the channel is formed by sequentially short segments that may span only part of the membrane, possibly as beta strands, was confirmed for potassium channels last year by mutagenesis experiments in several laboratories and has since been supported by additional experiments. This year our prediction that the homologous portion of sodium and calcium channels forms the ion selective region has been confirmed by mutagenesis experiments in which the ion permeation properties of a sodium channel were converted to those of a calcium channel by mutating two residues in the sodium channel to the residues that occur in the calcium channel. We have begun to extend our models to additional homologous channel proteins. As part of our homology analysis we determined that a newly sequenced putative potassium channel gene is more closely related to cylic nucleotide-gated cation channels than to voltage-gated potassium channels. This relationship had not previously been detected.

Our models of the influenza virus membrane fusion was developed farther to include more steps in the rather complicated process. To model this process, we modeled the transmembrane portion of the influenza hemagluttin (HA) protein onto the water soluble portions that has been determined by X-ray diffraction. We then examined how adjacent HA trimers could interact with each other and the viral and target membranes to induce the fusion process. These models have led to new proposals about how the 'fusion peptide' portion of the HA protein interacts with the membrane to induce transition states that lead to fusion. We continue to interact with Robert Blumenthal's group to develop experimental tests of the models.

Publications

Pollard HB, Rojas E, Pastor RW, Rajas EM, Guy HR, Burns AL. Synexin: Molecular mechanism of calcium-dependent membrane fusion and voltage-dependent calcium channel activity. Calcium entry and action at the presynaptic nerve terminal. Ann NY Acad Sci 1991;635:328-351.

Resnick NM, Maloy WL, Guy HR, Zasloff M. A novel endopeptidase from Xenopus that recognizes alpha-helical secondary structure. Cell 1991;66:541-554.

Guy HR, Durell SR, Warmke J, Drysdale R, Ganetzky B. Similarities in amino acid sequences of Drosophila eag and cyclic nucleotide-gated channels. Science 1991;254:730.

Durell SR, Guy HR. Detailed structural and functional models of voltage-gated potassium channels. Biophys J 1992;62:243-255.

Guy HR, Durell SR, Schoch C, Blumenthal R. Analyzing the fusion process of influenza hemagglutinin by mutagenesis and molecular modeling. Biophys J 1992;62:113-116.

Lazarovici P, Edwards C, Raghunathan G, Guy HR. Secondary structure, permeability and molecular modeling of Pardaxin pores. J Nat Toxins 1992;1:1-15.

Pollard HB, Guy HR, Arispe N, de la Fuente M, Lee G, Rojas EM, Pollard JR, Srivastava M, Zhang-Keck Z-Y, Merezhinskaya N, Caohuy H, Burns AL, Rojas E. Calcium channel and membrane fusion activity of synexin and other membranes of the Annexin gene family. Biophys J 1992;62:19-22.

Cruciani RA, Barker JL, Durell SR, Raghunathan G, Guy HR, Zasloff M, Stanley EF. Magainin 2: A natural antibiotic from frog skin forms ion channels in lipid bilayer membranes. Eur J Pharmacol, in press.

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

PROJECT NUMBER
Z01 CB 08366-09 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Pharmacology of Monoclonal Antibodies and Other Biological Ligands

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

John N. Weinstein,M.D., Ph.D. Chief, Theoretical Immunology Sect. LMMB, NCI

Other Professional Personnel:

William van Osdol, Ph.D.	Staff Fellow	LMMB, NCI
Jun Sato, M.S.	Guest Researcher	LMMB, NCI
Hitoshi Sato, Ph.D.	Visiting Fellow	LMMB, NCI
Toshiro Heya	Special Volunteer	LMMB, NCI

COOPERATING UNITS (if any)
LP, DCBDC, NCI; LAS, DCRT; LTIB, DCBDC, NCI; ROB, DCT; NMD, CC

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Theoretical Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.3	2.3	0.0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

Before a monoclonal antibody (or other biological ligand) can label or kill a tumor cell, it must generally reach that cell. For portions of a tumor more than a few microns from the nearest blood vessel, access may be limited by the rate at which the molecule can "percolate" through the extracellular space. We are investigating the spatial and temporal profiles of immunoglobulin (Ig) distribution generated by diffusion and convection through tumors, taking into account specific binding, nonspecific binding, and metabolism. For this purpose, we developed theoretical models that splice together the global pharmacology and the microscopic percolation process. Significant predictions: (1) Antibody molecules may be prevented from penetrating a tumor by the very fact of their successful binding to antigen (the "binding site barrier"). Thus, lower affinity might sometimes be preferable. (2) Even with saturable binding (but not metabolism), the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. (3) Metabolism will decrease the relative "C times T" exposure of cells farther from the blood vessel. This may be a major barrier to effective treatment of solid tumors with ligand molecules.

Predictions of the model have been tested using subcutaneous tumors and micrometastases in guinea pigs. We used a combination of double-label autoradiography and double-chromophore immunohistochemistry to determine simultaneously the microscopic distribution of antibody, isotype matched control IgG, antigen, and blood vessels. The result in both S.C. tumors and micrometastases was direct experimental validation of our "binding site barrier" hypothesis.

We have speculated that the "binding site barrier" is a factor in the evolution of physiological autocrine/paracrine and endocrine molecules. As a corollary, we think that the micropharmacology should be taken into account as we design the next generation of such molecules for exogenous administration or for secretion by genetically modified cells.

Project Description

Major Findings:

(1) For physiologically reasonable ranges of parameters, the diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor antibodies through tumors; (2) The flux of non-binding control antibody is much less likely to be limited by the rate of diffusion or convection through the tumor. Such antibodies are predicted to penetrate more deeply and more quickly into the tumor; (3) In the presence of saturable binding but not metabolism, the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. However, the period of exposure will be later for cells farther from the source of antibody; (4) Metabolism will decrease the relative CxT exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules; (5) Antibodies of low affinity may be preferable to those of high affinity at a given dose for some purposes, e.g. in therapy with alpha-emitting conjugates or toxin conjugates. (6) Quantitative, but not qualitative, changes in predicted behavior are seen as the geometry changes from Cartesian to cylindrical (for cords of tumor cells surrounding a central blood vessel) to spherical (for flux into a nodule of cells); (7) the characteristics for IgG, F(ab'), and Fab can been compared; (8) Bivalent binding can be, simulated; (9) the global pharmacology can be integrated with the percolation calculations; (10) the computer program package developed ("PERC") is broadly capable of handling various ligands of differing valence; (11) the "PERC-RAD" program was developed to calculate the radiation dose distributions resulting from spatial concentration distributions determined by PERC results. Calculations predict surprisingly great inhomogeneity for modules such as those of nodular lymphoma treated by I-131 immunoconjugates; (12) the "binding site barrier hypothesis" has been validated in both subcutaneous nodules and micrometastases of a guinea pig tumor.

AIDS Research:

This work pertains to AIDS in that it focuses on general principles of the pharmacology of antibodies--which are important in the immunology and therapy of AIDS, whether one is considering endogenous or exogenously administered antibody. Insofar as the principles delineated by this project apply to other classes of biological ligands (e.g., the lymphokines and cytokines), this work defines issues of basic science that are highly relevant to AIDS.

Publications:

Mulshine JL, Carrasquillo JA, Weinstein JN, Keenan AM, Reynolds JC, Herdt J, Bunn PA, Sausville E, Eddy J, Cotelingam JD, Perentesis P, Pinsky C, Larson SM: Direct intralymphatic injection of radiolabeled 111-In-T101 in patients with cutaneous T-cell lymphoma. Cancer Res 1991;51:688-695.

Van Osdol W, Fujimori K, Weinstein JN. An analysis of monoclonal antibody distribution in microscoppic tumor nodules: Consequences of a "binding site barrier." Cancer Res 1991;51:4776-4784.

Hui TE, Fisher DR, Press OW, Eary JF, Weinstein JN, Badger CC, Bernstein ID. Localized beta dosimetry of 131I-labeled antibodies in follicular lymphoma. J Biomed Eng, in press.

Weinstein JN, Van Osdol W. Early intervention in cancer using monoclonal antibodies and other biological ligands: Micropharmacology and the "binding site barrier". Cancer Res, in press.

Betageri GV, Black CDV, Szebeni J, Wahl LM, Weinstein JN. Fc-receptor mediated targeting of antibody-bearing liposomes to human monocyte/ macrophages. J Pharm Pharmacol, in press.

Weinstein JN, Van Osdol W. The macroscopic and microscopic pharmacology of monoclonal antibodies. Intl J Immunopharmacol, in press.

Weinstein JN. Immunolymphoscintigraphy in the diagnosis of lymphoma and solid tumors. Lymphology, in press.

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

PROJECT NUMBER
Z01 CB 08370-09 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Interactions in Globular Proteins and Protein Folding

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

Robert Jernigan, Ph.D. Deputy Chief, LMMB, NCI

Other Professional Personnel:

Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI
Peter Greif, M.D.	Computer Prog. Anal.	LMMB, NCI

COOPERATING UNITS (if any)
David Covell, BSC, PRI, FCRDC.

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

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

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

A novel approach has been taken to the problem of protein folding that examines the complete range of folded topologies accessible in the compact state of globular proteins. The procedure is to generate all conformations, with volume exclusion, upon a lattice in a space restricted to the individual protein's known compact conformational space. The importance of knowing with certainty the range of viable protein conformations is a compelling issue. The present studies have aimed at a more thorough evaluation of protein folds, with less than atomic detail. The concept that the overall chain tracing is more important than the precise positioning of each atom has been the basic assumption. Such atomless structures can be evaluated with potential function that are basically pairwise residue-residue hydrophobicities. Other aspects being considered include: folding intermediates, combining the folding calculations with sequence homologies and investigating hydrophobic cores, binding, choices of overall shapes and the relationship between good packing and secondary structures. The residue potentials are being generalized for application to situations with different solvent conditions.

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 the development of higher order principles of molecular structure is essential if we are to achieve a complete understanding of all of the complexities of biological macromolecules themselves, as well as their interactions with other small molecules, other macromolecules and their assembly into biological structures. This project is aimed in this latter direction.

We have collected statistics on globular proteins from their X-ray structures, counting the amino acid residues that are frequently found near one another in the three dimensional structures. These were obtained in the following way: a lattice model is used in which each residue type has a coordination number. If a specific residue has an incompletely filled coordination shell, then it is assumed to be filled with equivalent water molecules. These derived contact energies follow intuition, with the most favorably interacting pairs being hydrophobic residues. Also, they have a strong environment dependence that has been separately considered. This type of energy has a direct bearing on folding and substantially reflects the effects of water on folding. There is a segregation of hydrophobic and hydrophilic residues. These values reflect the actual situation inside proteins and provide a tool that can be applied to a variety of problems and, in the problem at hand, can be used to assess the relative overall quality of different conformations.

The present examples of generating all possible compact conformations on 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 then permits detailed examination of local packing arrangements that favor interactions of side-chains within the protein's interior. By using this relatively coarse-grained approach for examining conformational space and by subsequently adding atomic details onto this model, it should become possible to examine the role of amino acid sequence on three-dimensional structure. We have begun additional studies in order to develop approximate methods for larger proteins.

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.

Also these methods are being applied to the protein design problem to consider prediction of peptide binding sites on the surfaces of proteins.

Publications:

Ben-Naim A. The role of hydrogen bonds in protein folding and protein association. J Phys Chem 1991;95:1437-1444.

Jernigan RL, Covell DG. Compact protein conformations. In: Renugopalakrishnan V, Carey PR, Smith ICP, Huang S-G, Stover AC, eds. Proteins: structure, dynamics and design. Leiden, Netherlands:ESCOM Science Publishers, 1991:346-351.

Jernigan RL. Protein folds. Curr Opin Str Biol 1992;2:248-256.

Jernigan RL. Generating general shapes and conformations with regular lattices, for compact proteins. In: Sarma RH, Sarma MH, eds. Structure and Function: Proceedings of Seventh Conversation in Biomolecular Stereodynamics. Guilderland, New York:Adenine Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08371-09 LMMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Conformational Variation of DNA and DNA-Protein Binding

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

Robert Jernigan, Ph.D.	Deputy Chief, LMMB, NCI	
Other Professional Personnel:		
Gopalan Raghunathan, Ph.D.	Visiting Associate	LMMB, NCI
Victor Zhurkin, Ph.D.	Visiting Scientist	LMMB, NCI
Shou-ping Jiang	Visiting Scientist	LMMB, NCI
Brooke Lustig, Ph.D.	IRTA Fellow	LMMB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI

COOPERATING UNITS (if any)

Dr. Ruth Nussinov, Inst. of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; Dr. Jacob Mazur, and Dr. J.H. Chen, PRI, Frederick, MD; Akinori Sarai, RIKEN Institute; (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:
4.2	4.2	0.0

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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.)

Conformational analyses of DNA show that the origin of the specific type of double helix depends mainly on the stacking interactions with its neighboring bases. In addition to sequence specific preferences for mean positions of stacking there are also substantial sequence specific dependences in the conformational fluctuations. Both effects manifest themselves in the bending behavior. To investigate these effects Monte Carlo samples of DNA double helical conformations have been generated. 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 cut data. Such calculations depend in detail on the way in which the electric interactions in the DNA polyelectrolyte chain are treated. A new approach for treating the dielectric function was developed and favorable results were found that indicate the type of helix taken by different sequences.

Ways to treat RNA folding in three dimensions were considered. Transfer RNA was used as a test molecule to investigate large numbers of possible arrangements. In the largest generation of such conformations, over 2 million, several types of variant conformations were observed. There was some flexibility in the anticodon loop and several cases of "slip pairing with a single base bulge". This later case requires two identical bases at the helical boundary on one strand. For example, for sequential bases a,b,c,d,e, and f on one strand and D,C,B,E and F on the other, where a can pair with A, b with B, etc., this transformation corresponds to passing from

```
abbcd          ab b cd
 •••     to     •  ••   where dots indicate base pairs.
FEBCD          FEB CD
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Cooperating Units (Continued):

Daniel Camerini-Otero, NIDDK; H. Todd Miles, NIDDK; V. Sasisekharan, NIDDK.

PROJECT DESCRIPTION

Major Findings:

A static picture of DNA double helical conformations is only a first approximation. In addition there can be substantial flexibility through 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 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.

A new dielectric function was formulated using a Langevin function to span the region of dielectric between bulk solvent and cavity. In calculations, DNA double helical conformations are unusually sensitive to the form of the dielectric function, just as they are experimentally sensitive to solvent conditions such as ionic strength. With this new formulation, it was possible to demonstrate the sensitivity of certain base sequences to take either A or B helix forms. Details of the base stacking are more sensitive to the form of this function than are the sugar-phosphate backbone conformations. Also, in the B-form helix, the local backbone conformation is not so directly affected by the attached base as in the A-form. In this sense, the B-form helix is more regular and less sensitive to the specific sequence; this relative independence of sequence could be related to the required environmental conditions during the biological transfer of information from the DNA.

Conformations of the 76 nucleotide phenylalanine t-RNA have been studied, based on its known crystal structure. 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 in this list prevents the direct generation of all possible base pairs. It was found that the use of secondary structures was necessary to bring the calculations into feasible range. After a set of secondary structures were found, 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 surprising and implies that such folding approaches will require large preliminary reductions, such as secondary structure approaches.

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 has been proposed on the basis of fiber X-ray and molecular modelling. Models for protein-nucleic acid interactions have been proposed, including that with rec A, for recombination.

Publications:

Mazur J, Jernigan RL. The distance-dependent dielectric constants and their application to double-helix DNA. Biopolymers, 1991;31:1615-1629.

Zhurkin VB, Ulyanov NB, Gorin AA, Jernigan RL. Static and statistical bending of DNA evaluated by Monte Carlo simulations. Proc Natl Acad Sci USA, 1991;88:7046-7050.

Templeton NS, Rodgers LA, Levy AT, Ting K-L, Krutzsch HC, Liotta LA, Stetler-Stevenson WG. Cloning and characterization of a novel human cDNA that has DNA similarity to the conserved region of the collagenase gene family. Genomics, 1992;12:175-176.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08380-08 LMMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Molecular Structure of Animal Viruses and Cells by Computational Analysis

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

Jacob V. Maizel, Jr., Ph.D. Chief, Laboratory of Mathematical Biology, LMMB, NCI

Other Professional Personnel:

John Owens	Computer Specialist	LMMB, NCI
Lewis Lipkin, M.D.	Medical Officer	LMMB, NCI
Ann Barber, M.D.	Guest Researcher	LMMB, NCI
Bruce Shapiro, Ph.D.	Computer Specialist	LMMB, NCI

COOPERATING UNITS (if any)

Dr. Danielle Konings, Univ. of Colorado, Boulder, Colorado, Dr. Ruth Nussinov, PRI, NCI/FCRDC, Frederick, Maryland, and Sackler Institute of Molecular Medicine, Israel; Dr. Hugo Martinez, Consultant, PRI, NCI/FCRDC, Frederick, Maryland.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892 and Frederick, MD 21702

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.2	3.2	1

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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.)

Complex macromolecular processes and the structural organization of normal, infected and transformed cells are modeled using viral systems. Computers are used to study nucleic acid and protein sequences that embody the information of living systems.

Computer analyses of proteins and nucleic acids are developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, and human immunodeficiency viruses. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Computer programs are developed locally and elsewhere for application on vector and massively parallel supercomputers, minicomputers and graphic workstations to perform sequence analysis and structure predictions. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Roles for genes and proteins are deduced by comparison with databases of sequences of known function and structure.

PROJECT DESCRIPTION

Major Findings:

Multiple sequence alignment for the detection of signals: Multiple sequences for related genes provide a powerful data set for detection of the structural entities involved in recognition by enzymes or regulatory elements, and for understanding common structural motifs. Programs reported last year were refined and continue to be applied to pattern detection problems. SEA (Sequence Editor and Aligner), formerly called ANA, detected consensus sequences in prokaryotic CAP-binding DNA with internal spacers of defined length reminiscent of bent DNA. Experimental studies and structural modeling supported the hypothesis (Barber, Zhurkin, Adhya). The FEATUR(x) programs for fixed-point multiple alignment were translated from Pascal to C and adapted to Unix systems thereby making them more widely portable in the scientific community (Owens). Efforts to develop further theoretical foundations for sequence analysis and to detect putative functional domains in unannotated sequences progressed with discovery of information-theoretic principles for the distribution of short oligonucleotides. This and other aspects of what may be called the "biological coding problem" were the focus of an international workshop (Konopka).

Similarity studies: Sequence comparison is one of the most powerful techniques for understanding the organization and function of biological systems. Continuing development and testing of methods of sequence comparison are needed. Standards of performance are lacking and need to be developed. With the recent availability of adequate amounts of mass storage at the upgraded Biomedical Supercomputing Center work has begun to assess the relative merits of exhaustive methods versus fast methods employing intuitive rules. Very efficient and popular programs such as BLAST(x) and FAST(x) are being compared with highly optimized dynamic programming using SEQH(x) programs of Kanehisa. A recent optimization by Cray computer scientists using a technique called "pocket arithmetic" allows 3-5X speedup for our SEQHDP program that gives local alignment with gaps and randomization statistics. Early versions of dynamic programs on the MasPar massively parallel computer contribute to the qualitative sense that detailed methods find a higher fraction of known members of large, test protein superfamilies with comparable performance speeds to "FAST" algorithms. A new effort in the laboratory aims at developing three-dimensional structure comparison methods with efficiency comparable to sequence comparisons. Using computer science methods developed for robotic/computer vision applications Nussinov, with collaboration of Wolfson and others of Tel Aviv University succeeded in recognizing known 3-D motifs automatically and independently of the amino acid sequence. This method has promise in gaining insight into the classes of structures based on interior (skeletal), volume and surface characteristics. Further goals of the project are to incorporate chemical properties and to adapt the method to docking between complementary molecular surfaces. The speed of these programs is such that these otherwise very intensive computations may now be afforded.

Detection of potential RNA structure: RNA and DNA structure are of increasing concern as the innate catalytic activity, and other functional properties of polynucleotides are recognized and more experience with protein-nucleic acid interactions accumulates. Evidence from simple systems suggest that primary sequence determines the regions of helical base pairing and stacking that pack into 3-dimensional structures in a hierarchical order. Reasonable predictions of the secondary structure are needed in predicting the full structure. Extensions and modifications were made to RNA folding programs for further understanding of structure prediction techniques. Energy parameter sections were generated to simulate natural experimental errors to test the ability to predict common structure in the RNA, a critical test of any folding program. Using stringent criteria correct structures were located within a very small deviation from "standard" values. Structure generated this way can be used in an attempt to find a consensus for RNA families, or to produce a single, refined set of parameters for broader use. The efficacy of this method is at least comparable to known ones for finding multiple near optimal structures that agree with experimental data. It is undergoing further refinement and testing. A program for finding pseudoknots of unusual structure locates such features in T4 phage gene 60 and in poliovirus RNA in regions correlated with biological functionality (Le and others). Functions of RNA molecules, as with proteins, are manifestations of 3-D structure. A program by Martinez is nearing completion to permit folding secondary structure models into three dimensions. Output from the program can be directed to molecular mechanical programs for refinement based on atomic and geometrical principles. It is highly interactive and fast yet capable of producing plausible models.

Publications:

Le S-Y, Shapiro BA, Chen J-H, Nussinov R, Maizel JV. RNA pseudoknots downstream of the frameshift sites of retroviruses. Gene Anal Tech and Applic 1991;8:191-205.

Nussinov R. Compositional variations in DNA sequence. Comput Appl Biosci 1991;7:287-293.

Nussinov R. Distinct patterns in the dinucleotide nearest neighbors to G/C and A/T oligomers in eukaryotic sequences. J Mol Evol 1991;33:259-266.

Nussinov R. Long range and symmetry considerations in the DNA. DNA Sequence 1991;2:69-79.

Nussinov R. Signals in DNA sequences and their potential properties. Comput Appl Biosci 1991;7:295-299.

Nussinov R, Smythers G. Trends in the 5′ vs. 3′ flanks of oligonucleotides in eukaryotic and prokaryotic genomes: The asymmetric roles played by cytosine and guanine. J Theor Biol 1991;153:111-135.

Nussinov R, Wolfson HJ. Efficient detection of three-dimensional structural motifs in biological macromolecules by computer vision techniques. Proc Natl Acad Sci USA 1991;88:10495-10499.

Dayton ET, Konings DAM, Powell DM, Shapiro BA, Butini L, Maizel JV, Dayton AI. Extensive sequence specific information throughout the CAR/RRE, the target sequence of the HIV-1 rev protein. J Virol 1992;66:1139-1151

Fischer D, Bachar O, Nussinov R, Wolfson H. An efficient automated computer vision based technique for detection of three dimensional structural motifs in proteins. J Biomol Struct Dyn 1992;9:769-789.

Konings DAM, Nash M, Maizel JV, Arlinghaus RB. Novel GACG-hairpin pair motif in the 5' untranslated region of type C retroviruses related to murine leukemia virus. J Virol 1992;66:632-640.

Nussinov R. DNA spatial considerations in the arrangement of G/C and A/T blocks. Comput Biol Med 1992;22:97-112.

Nussinov R. The eukaryotic CCAAT and TATA boxes, DNA spacer flexibility and looping. J Theor Biol 1992;155:243-270.

Nussinov R. Intervening looped sequences between CCAAT and TATA in primates are rigid and upstream and flexible downstream. Structure and Function 1992;1:183-212.

Chen J-H, Le S-Y, Maizel JV. A procedure for RNA pseudoknot prediction. Comput Appl Biosci, in press.

Le S-Y, Nussinov R, Maizel JV. Significant terminal RNA stem-loop structures in histone mRNA and U-snRNA and a feature for describing their statistical distribution. Adv in Math & Comp in Med, in press.

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

PROJECT NUMBER
Z01 CB 08381-09 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Computer Aided Two-Dimensional Electrophoretic Gel Analysis (GELLAB)

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

Peter F. Lemkin, Ph.D. Computer Specialist IPS, LMMB, NCI

Other Personnel:

Kyle Upton Scientific Applications Analyst PRI/FCRDC

COOPERATING UNITS (if any)
Dr. J. Myrick, CDC/Atlanta; Dr. P. Rogan, Penn State Med. Sch.; Dr. R. Levenson, Duke Med. Sch.; Dr. P. Sonderegger, T. Hale, Biochem. Inst., Univ. Zurich; Dr. T. Krekling, Agr. Univ. Norway; Mr. C. McGrath, (continued)

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	1.0	1.0

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

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

Analytic methods are continuing to be developed and applied using the GELLAB-II software system - an exploratory data analysis system for the analysis of sets of 2D electrophoretic gel images. It incorporates sophisticated subsystems for image acquisition, processing, database manipulation, graphics and statistical analysis. It has been applied to a variety of experimental systems in which quantitative and qualitative changes in one or more proteins among hundreds or thousands of unaltered proteins is the basic analytic problem. Keeping track of changes detected using these methods is also a major attribute of the system. A composite gel database may be "viewed" under different exploratory data analysis conditions and statistical differences and subtle patterns elucidated from "slices" of an effectively 3D database. Results can be presented in a variety of tables, plots or derived images and on workstations over wide area networks.

Substantive applications include: Ongoing studies of cadmium toxicity in urine (Myrick), Rett syndrome (Myrick), Vermont mercury study (Myrick), serum dioxin study (Myrick); 2D DNA gels for identifying differentially expressed genes (Rogan et al.); radioactive fall-out from Chernobyl (Krekling); Aids Vaccine Program/FCRDC using image processing to improve: quantitative analysis of HIV-1 protein gels and Western blot assays (McGrath et al.). GELLAB-II had been exported to: CDC/Atlanta (Myrick), Univ. Norway (Krekling), Univ. Zurich (Sonderegger). This year, CDC has been the primary Beta test site for GELLAB-II. Additional changes to help continued collaboration with and exporting of enhanced graphical-interface versions of GELLAB has been actively pursued and the primary activity this year.

Cooperating Units (Continued):

Dr. L. Arthur, AVP/FCRDC; Dr. Andrew Grimshaw, UVA.

PROJECT DESCRIPTION

Major Findings:

Two-dimensional gel electrophoresis analytic methods are continuing to be developed, enhanced and applied using the GELLAB-II system. A major effort was spent on simplifying and enhancing the user interface for GELLAB-II programs to use the X-windows X11 with the Xt toolkit and Xaw Athena widgets. These new graphical interfaces makes learning and using the system much easier.

The GELLAB-II programs have grown to over 240,000 lines of code and continues to be fine tuned to simplify the user interface and analysis. Part of this work was in preparation for an anticipated CRADA starting this next year. We have been negotiating and are submitting a CRADA with OTD/NCI to transfer the GELLAB-II technology to private industry so it can be commercialized. Such commercialization will result in wider use and better support than we can provide. A large effort was spent in studying the current status, defining what needs to be done in order to prepare it for commercialization, etc. We have also visited the projected Company in order to better evaluate their suitability for this CRADA.

This year, Kyle Upton (PRI/FCRDC) helped optimize the automatic spot pairing AUTOPAIR program (to automatically pair spots between two gels). We have added new algorithms to robustly find initial landmarks using a recursive 2-level local morpholgic neighborhood pair-quality measures. AUTOPAIR then uses these initial landmarks to pair all other spots in the gels. The pair-quality heuristics were extended to include more features so pair-quality is more robust and has better discrimination in pairing spots.

A difficult sub-problem in segmenting gels is handling touching clusters of very large saturated or near-saturated spots. In such cases, the Laplacian does not exist or is too weak to separate the spots. [This problem arose in analyzing some of the Urine gels from Dr. Myricks Denver study data.] So we extended the gel-spot segmenter algorithms for our 2D gel segmenter SG2GII. The new algorithm analyzes a merged spot cluster's boundary shape using the idea that touching spots define notches with small enclosing angles. By pairing the appropriate notches, the segmenter can then split the touching spots and then resegment them into separate sub-regions. This new algorithm handles an order of magnitude more of these difficult spots than the older method.

We have rewritten the 2D gel landmarking (LANDMARK11) and data acquisition programs (GETACC11) using the new X11 Xt toolkit and Athena widgets. LANDMARK11 has additional functionality which suggested itself from our collaboration with Dr. Myrick which gives the investigator more flexibility when editing 2D gel landmarks. Because converting gel images from a variety

of scanners is essential for portability, we rewrote the gel image file conversion program PPXCVT. It now provides a more consistent and flexible general gel image conversion model allowing us to use scanner data from non-standard scanners much more easily.

The reference manual "GELLAB-II Exploratory Data Analysis" discussing methods and usage has been expanded and updated.

Xconf Image Conferencing:

We have continued our research on using the Xconf image-conferencing system to aid collaborative research. Xconf was originally developed to explore remote collaboration of 2D gel images using workstations connected via national and international networks. Conferees may interactively point to and discuss image data with actions visible to everyone in the conference. This year we investigated the feasibility of, and problems in, using other types of images: molecular models (with Stewart Durrell/NCI) and MRI images (with Steve Bacharach, NCI/CC). This has led to improved functionality including the use of interactive movies. A paper describing this work has been submitted.

Collaborations:

Our primary collaboration this year has been with Dr. James Myrick (CDC/Atlanta). We have increased our collaboration investigating several large 2D gel databases focusing on a cadmium toxicity study (29 gels); a Rett syndrome study (77 gels) as well as studies of Mercury in urine and serum dioxin. We have also been able to experiment with image-networking using the Internet to share data. We have also been helping Dr. Myrick using the Internet to set up his system of four SUN computers to handle additional GELLAB collaborations with groups from the Morehouse School of Medicine. The four studies are include: (1) Denver Cadmium Study. This is a project involving NIOSH and CDC to determine the health effects of occupational cadmium exposure in a group of metal recovery plant workers. The Protein Electrophoresis Laboratory (Clin. Biochem. Br., Div. Env. Health Lab. Sci, Nat. Center for Env. Health and Injury Control, CDC) has analyzed the urine samples from these workers with a BioImage system to attempt to find a better protein biomarker(s) of cadmium toxicity using statistical analysis. The project has been extended to include analysis of the same images by the GELLAB-II system. (2) Rett Syndrome Study. Rett Syndrome is a unique mental retardation syndrome reported only in young girls (1:10,000) for which there are no known diagnostic markers. We are using quantitative 2-D electrophoresis to analyze sera from affected children and age-matched controls to create a gel-to-gel matched database of detectable proteins. Statistical analysis of the database will be used to search for any protein(s) that may be of diagnostic significance for Rett Syndrome. (3) Vermont Mercury Study. Thermometer factory workers in Vermont are the subjects of this study. Urine samples are being analyzed by 2-D electrophoresis, and the intensities of matched proteins will be correlated with known body burdens of Hg to find an early marker of Hg toxicity. (4) A Serum Dioxin Study. This project

involves persons with known body burdens of 2,3,7,8-TCDD (dioxin). Sera are being analyzed by 2-D, and matched, quantified proteins will be correlated with the serum dioxin concentrations. The health effects of low levels of dioxin are largely unknown in humans. Early serum protein changes may lead to further studies that could better describe the health effects from dioxin.

Trygve Krekling (Agric. Univ. Norway) has collaborated in extending the range of images which GELLAB-II can interpret. His was the first site which we were able to directly access over the Internet and this helped us develop strategies for updating user GELLAB-II systems with minimum effort. In addition, the Isotope and Electron Microscopy Laboratories use GELLAB-II in an ongoing investigation (started 1991) of radioactive fall-out material from Chernobyl and elsewhere. Soil samples from polluted sites are deposited on cardboard with sticky surfaces and autoradiographs made. Autoradiographs are analyzed, using GELLAB-II, for localization of radioactive particles and estimating their activity. Based on this, pieces of the cardboard (containing active particles) are cut out and prepared for scanning electron microscopy and X-ray analysis for determination of micro-structure and element composition. These particles, air carried, are very small (micron - nanometer range), and would have been hard to isolate from the 10,000's of other particles using other methods. 2) Particle size and micro-structure are important parameters concerning bio-availability since there is often an inverse relationship between size and bio-availability.

With Peter Rogan (Penn State Med. Sch.) we are continuing in using GELLAB-II to help analyze 2D DNA gels based on restriction enzymes to be used for monitoring active transcription sites in yeast and other systems. During the past year, two manuscripts describing the development of electrophoretic and analytical tools associated with the extrinsic methylation technique have appeared. We have successfully demonstrated that the introduction of a foreign methylase into yeast cells can reveal cell-type specific sites of genomic modification as unique spots on a two-dimensional electrophoretic grid. A third manuscript describing this technique, the biology of the methylation system, and these results is in preparation.

Peter Sonderegger will again be using GELLAB-II this summer in a way similar to that which was used successfully to identify axonin-1 (a protein that is secreted from axons of cultured chicken embryonic dorsal root ganglion neurons (Stoeckli et al. 1989). Axonin-1 protein was subsequently purified, cloned and its functionally characterized. The new project will compare different neuronal subpopulations with respect to additional proteins secreted from their axons. Preliminary evidence has been obtained from cultures of spinal cord neurons, that their secretory pattern is indeed different from that of dorsal root ganglia neurons. Other neuronal populations such as retinal and cerebellar neurons will be included.

Investigating MIMD computing for gel analysis:

We have also been investigating the use of MIMD computation (Multiple Instruction Multiple Data) for handling some of the compute-intensive parts of

the 2D gel computer analysis. We are involved in collaborations with two different groups in this work. The first, with Dr. Richard Levenson (Duke Med. Sch.), Paul Lanzkron (Comp. Sci./Duke Univ.) has resulted in the development of an interactive image segmenter (MARGO) incorporating some of our algorithms for segmenting giant gels of NIH-3T3 cells. The Giant gels are currently being used in two projects in Dr. Levenson's laboratory: 1) studying the effects on host protein synthesis of expressing the human T-cell leukemia virus I (HTLV-I) transactivating protein, tax; 2) defining the intracellular and secreted protein phenotype of reversibly immortalized IMR90 cells as they undergo a process of accelerated cellular senescence. The MARGO system runs on a network of SUN computers and has shown computational speedups of over 10:1.

The second MIMD-oriented collaboration is with Dr. Andrew Grimshaw (Comp. Sci. Univ. VA) who has developed the MENTAT language based on C++ for MIMD programming. Unlike MARGO, MENTAT is not restricted to a homogeneous network - it can use a mixed network SUNS and SGI systems. We have also installed it on the 8-processor BSC CRAY, but ran into some problems due to restrictions in UNICOS. It has been used successfully to program one of the key convolution algorithms necessary for 2D gel spot segmentation. Ongoing work will be spent in re-designing other parts of the segmentation algorithm to take advantage of the MIMD environment.

New collaboration for improving image processing methods: A collaboration has been initiated this year with Mr. Connor McGrath for Dr. Larry Arthur, AIDS Vaccine Program, (AVP/FCRDC). We are starting to collaborate on projects to use image processing to improve: quantitative analysis of HIV-1 protein gels for comparison of preparative techniques for Retroviral Protein Section (Dr. Lou Henderson); quantitative analysis of HIV Western blot assays in batch for Human Retrovirus Section (Dr. Dave Waters).

AIDS summary:

A collaboration has been initiated this year with Mr. Connor McGrath for Dr. Larry Arthur, AIDS Vaccine Program, (AVP/FCRDC). We are starting to collaborate on projects to use image processing to improve: quantitative analysis of HIV-1 protein gels for comparison of preparative techniques for Retroviral Protein Section (Dr. Lou Henderson); quantitative analysis of HIV Western blot assays in batch for Human Retrovirus Section (Dr. Dave Waters).

Publications:

Lemkin PF, Rogan P. Automatic detection of noisy spots in two-dimensional Southern Blots. App Theoretical Electrophoresis 1991:141-149.

Rogan P, Lemkin PF, Klar A, Singh J, Strathern JN. Two-dimensional agarose gel electrophoresis of restriction-digested genomic DNA., Methods: A Companion to Methods in Enzymology 1991;3:91-97.

Amberger A, Tontsch U, Lemkin PF, Gabbiani G, Bauer HC. Two cloned cerebral endothelial cell phenotypes: An in Vitro model for angeiogenesis. In: Steiner R, Weisz PB, Langer R, eds. Angiogenesis: Key Principles - Science - Technology - Medicine. Basel: Birkharuser Verlag; 1992;2:244-249.

Lemkin P. Representations of protein patterns from 2D gel electrophoresis databases. In: Pickover C, ed. The visual display of biological information. Teaneck, New Jersey:World Scientific, in press.

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

PROJECT NUMBER
Z01 CB 08382-09 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

Bruce A. Shapiro, Ph.D.	Computer Specialist	LMMB, NCI
Other Personnel:		
Joseph Navetta	Computer Programmer	LMMB, NCI
Kathleen Currey, M.D.	Guest Researcher	LMMB, NCI
Morton L. Schultz	Electronics Engineer	LMMB, NCI
Jacob V. Maizel, Jr., Ph.D.	Chief, Lab. of Math. Biol.	NCI

COOPERATING UNITS (if any)
Andrew Dayton, Ph.D., LIR, IDIR; Danielle Konings, Ph.D., Univ. Colorado; David Friedman, Ph.D., Dept. of Microbiology and Immunology, Univ. Michigan; Eric Baldwin, Ph.D., PRI; Wojciech Kasprzak, Applications Analysts, PRI, (continued)

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:
2.7	2.0	0.7

CHECK APPROPRIATE BOX(ES)
☐ (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.)

Work has begun exploring a relatively new class of algorithms, "genetic" algorithms, to facilitate the modeling of molecular structures, specifically currently RNA molecules. These algorithms have been shown in several applications to be parallelizable and rapidly convergent to solutions in a large search space consisting of many possible suboptimal results.

It is partially as a result of this research that much effort over the past year has been spent in investigating the various forms of massively parallel computer architectures to determine which offers the best performance and computational environments. This has resulted in the acquisition of an 8000 processor MasPar computer system.

We are doing comparative studies with a newly developed sequence matching program based on the Smith/Waterman algorithm that runs on the MasPar. This algorithm is capable of performing 50,000,000 comparisons per second on a 4000 processor system. The results from these runs are being compared with other algorithms including another new algorithm running on a systolic array.

The genetic algorithm and the MasPar architecture have been integrated into the heterogeneous system that is being developed for RNA structure analysis. This system includes the facilities for activating algorithms that may run on various computer architectures that are accessible over a computer network.

The termination structures of lambda TR2 have been extensively studied and the 5' non-coding regions of Polio-virus and its mutational relationships to RNA secondary and tertiary structures are being characterized in regard to its functionality.

The system has been used to study the fine structural details of the HIV-1 rev responsive element (RRE) and is currently being used to study the dimerization structure of HIV-1.

Cooperating Units (Continued):

and Dzung Hoang, PRI, FCRDC.

Project Description

Major Findings:

At the present time research is being pursued in the development of new algorithms and software to facilitate the modeling of nucleic acids, specifically RNA molecules. This includes the exploration of a class of algorithms known as "genetic" algorithms (with Joseph Navetta) which have shown in several applications to be parallelizable and rapidly convergent to solutions in a large search space consisting of many possible suboptimal results.

Thus, our goal in this project is to investigate representations that are appropriate for such "genetic" algorithms so that they may be applied to the problem of predicting RNA secondary and tertiary structure. Such predictions are then to be compared with the performance of currently used algorithms both from the standpoint of accuracy of predictions as well as speed. The algorithms are to be developed with massively parallel computing architectures in mind. This includes the MasPar MP-1.

These algorithms have been integrated into a system for RNA structure analysis that is being developed. This system includes the facilities for activating algorithms that may run on various computer architectures that are accessible over a computer network.

The purpose of this work is to help to further the field of molecular modeling by examining a potentially fruitful new algorithmic approach that permits a relatively efficient means of exploring a very large search space of molecular conformations. These approaches may be applicable to other molecular modeling domains such as protein folding. The development of such algorithms permit a very close interaction between experimentalists and computational biologists in helping to define approaches to understanding the mechanisms of molecular function.

Partially because of the highly parallel nature of these algorithms much time has been spent on investigating various forms of massively parallel computer architectures including MIMD (multiple instruction multiple data) and SIMD (single instruction multiple data) paradigms (with Joe Navetta, Jacob Maizel). It was determined that at this point in time the SIMD paradigm was most mature and offered the best computing environment. This led to the purchase of a 8000 processor MasPar with an associated disk array.

The current version of the genetic algorithm for RNA secondary structure prediction running on the MasPar generates 8000 structures per generation. At the current time we appear to be getting exponential convergence to structures that look reasonable although more experiments need to be done. Testing has

included various selection algorithms used with the "crossover" and "mutational" aspects of the genetic algorithm.

In addition, we have been examining the results from a new sequence comparison algorithm, developed by MasPar, based upon the Smith/Waterman algorithm that runs on the MasPar (with Jacob Maizel and Joseph Navetta). This algorithm is capable of doing 50,000,000 exhaustive comparisons per second on a 4000 processor system. These results are being compared with other sequence comparison algorithms. It is currently felt that massively parallel systems constitute the only efficient way to compare the large quantities of data that will result from the sequencing of the human genome.

Also, a new systolic algorithm was implemented on SPLASH (with Dzung Hoang, Mort Schultz) which not only computes edit distances but also includes alignment information. Because of the extensive pipelining in the systolic array, computing an alignment on SPLASH takes the same amount of time as computing the edit distance. Compared to conventional computers, SPLASH performs several orders of magnitude faster. At the current time a new systolic array configuration, SPLASH II, is being developed at the Super Computing Research Center which should substantially enhance SPLASH's performance both in its computational and I/O capabilities. Eventually the sequence comparison algorithm will be moved to SPLASH II. This approach may eventually lead to a relatively inexpensive mechanism for doing rapid extensive sequence comparisons with a plug in board to a standard workstation.

An optimal version of Zuker's RNA folding code has been ported to the MasPar (with MasPar). This permits the folding of an RNA sequence in times which are proportional to the 1.7 power of the length of the sequence as opposed to more conventional architectures with running times proportional to the 2.7 power. On a 16000 processor MasPar it is possible to fold HIV (~9300 nucleotides) in 5.5 hours. This was accomplished with little attention paid to further optimizations. Thus, even better speeds can be expected.

The MasPar with the currently running version of the genetic algorithm for RNA structure prediction has been incorporated into the heterogeneous computing environment for the analysis of RNA secondary and tertiary structure that runs with a front end SUN workstation. One of the objects of this research is to make available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA secondary and tertiary structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system is forming the basis for an expert system which is permitting intelligent queries of relationships that exist in the RNA secondary and tertiary structure problem domain involving various software/ hardware complexes available at the Frederick Cancer Research and Development Center and elsewhere. The system currently has a large number of functions that permit the use of algorithms that reside on different nodes within and external to the FCRDC network. This includes the SUN'S, SILICON GRAPHICS, CONVEXes, VAXES, CRAY and the MasPar. These algorithms are invoked from the SUN utilizing one common mouse and window system that reduces the users need to know the various software/hardware complexes. The user has the interactive

capability to fold literally hundreds of structures and to cluster 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 significance algorithm and display its results to determine regions of RNA that have potentially interesting structures. The ability exists to graphically display structures that are generated. One may then interact with the display to get at various local structural elements. The structure may be labeled in different ways so that the important area of current interest may be viewed. Structures may be compared analytically, for example using a Boltzman distribution as well as visually. The system is in the process of being enhanced to include more functionality (with Wojciech Kasprzak) thus allowing broader access to the research community. The SUN version of the system, which is currently being utilized by selected individuals, utilizes X-windows which permits the running of the system from many different types of workstations across networks i.e. one can start up the system in the United States and interact with a display in Europe. The system has been used from various SUN workstations as well as Silicon Graphics workstations.

Algorithms and methodologies have been developed that permit the clustering, searching for, and examining in detail of multiple RNA secondary structures from a structural as well as sequence standpoint. The object is to find structural similarities that could be correlated with functional sites. This includes the examination of how mutations affect structure and function in RNA. Mutations may be generated that preserve the amino acid coding sequence and then these structures are then compared to determine which ones are similar to the wild type or other alternate structures. These in turn suggest mutational experiments to perform in the laboratory to determine how the mutants perform in comparison with the predicted models. New mutational algorithms have been added that permit the exploration of mutational space when looking for arbitrary mutations that conserve specified substructures. These structure specific mutations have then been used to indicate to experimentalists sequences to generate in the laboratory that should exhibit suspected functionality.

Computer and biological experiments have been pursued in studying the lambda TR2 terminator (see 1). Specifically we report in vivo studies showing that single nucleotide changes reducing potential RNA stem stability eliminate TR2 activity and multiple changes in the stem sequence that should not affect stability of the stem, significantly reduce terminator activity. This suggests that for the TR2 terminator the stem structures may have multiple roles providing both structure as well as sequence specificity to signal transcription termination (with Sheau-Wei Chang-Cheng, Eileen Lynch, Kenneth Leason, Donald Court and David Friedman).

A study of potential RNA pseudoknots just downstream from known and suspected retroviral frameshift sites has been done (see 2). For each sequence, the thermodynamic stability and statistical significance of the secondary structure involved in the predicted tertiary structure are assessed and compared. Our results show that the stem-loop structure in the pseudoknot is

both highly stable and statistically significant relative to others in the gag-pol or gag-pro junction domains. Phylogenetic studies were also done which show the occurrence of compensatory base changes in the homologous stems of these related sequences which allow the conservation of the tertiary structures despite sequence divergence (with Shu-Yun Le, Jih-H Chen, Ruth Nussinov and Jacob Maizel).

Work has continued on a methodology to predict pseudoknots from RNA secondary structures. This is based on the premise that most pseudoknotted structures which have been reported in the literature appear to be derivable from their "precursor" secondary structures. Our approach uses a combined method of comparing potential pseudoknots generated without regard to secondary structure with secondary structural elements (with Danielle Konings).

Collaborative efforts (with Kathleen Currey) has continued in looking at the 5' non-coding region of Polio-virus RNA. We are examining the effects of single random mutations and the likelihood of whether they will result in local or global rearrangements in structure. Using structural comparison techniques, provided for in the RNA structure analysis system, we are able to group the structural rearrangements. We have observed global rearrangements, local rearrangements and no changes in structure. We have also noted that distant separate mutations may result in similar structures. In looking at these more closely, we noted that these seem to have as expected, mutations in loops, single stranded regions and in helical stems with compensatory base changes (i.e. GU to GC or vice-versa). It has been shown that single base changes can determine the switch from neurovirulence to non-neurovirulence and this has been related to structure (Skinner et. al). We are looking at these specific sites as well as others where we have data showing that mutations (insertions and deletions) affect function (Trono et. al.). Our purpose is to examine and characterize structure/function relationships using our mutational data as a guide.

Computer and biological experiments have been pursued in refining and understanding the structure of the rev responsive element (RRE) of HIV-1. This has included the designing of mutants based upon computer computations of structures to determine what structural as well as sequence elements are required for activity as well as protein binding of the RRE. A comprehensive experimental and computational approach combining mutational analysis, phylogenetic comparison and thermodynamic structure calculations with a systematic strategy for distinguishing sequence specific from secondary structural information was used. A target sequence analogue was designed to have a secondary structure identical to the wild type but a sequence that differed from the wild type at every position. This analogue was inactive. However, by exchanging fragments between the wild type sequence and the inactive analogue we were able to detect an unexpectedly extensive distribution of sequence specificity throughout the CAR/RRE. In addition an important sequence specific base paired region (called IIb) in the REV binding domain has been determined (see 3) (with Elahe Dayton, Andrew Dayton, Danielle Konings, Douglas Powell, Luca Butini and Jacob Maizel).

Work has begun (with Eric Baldwin) on determining the structure of the HIV dimer structure and nucleocapsid binding. This has included the use of the RNA structure analysis system to determine potential secondary structures and mutants for testing out various structural hypothesis. This information is then being utilized to build a three-dimensional structure of the dimer region.

Publications:

Cheng S-W C, Lynch EC, Leason KR, Court DL, Shapiro BA, Friedman DI. Functional importance of sequence in the stem-loop of a transcription terminator. Science 1991;254:1205-1207.

Le S, Shapiro BA, Chen J-H, Nussinov R, Maizel JV. RNA pseudoknots downstream of the frameshift sites of retroviruses. Gene Anal Tech and App 1991;8:191-205.

Dayton ET, Konings DAM, Powell DM, Shapiro BA, Butini L, Maizel JV, Dayton A. Extensive sequence-specific information throughout the CAR/RRE, the target sequence of the human immunodeficiency virus type 1 REV protein. J Virol 1992;66:1139-1151.

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

PROJECT NUMBER
Z01 CB 08387-05 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

Pedro Pinto da Silva, Ph.D.	Chief, Structural Biology Section	LMMB, NCI
Other Professional Personnel:		
Ru-Long Shen, M.D.	Guest Researcher	LMMB, NCI
Wan-Ying Hou, M.D.	Visiting Fellow	LMMB, NCI
Hector Caruncho, Ph.D.	Guest Researcher	LMMB, NCI
Cristina Risco, Ph.D.	Post Doctoral Fellow, Min. of Ed., Spain (G.R.	PRI/FCRDC)
Eliana Munoz, M.T.	Research Associate	PRI/FCRDC
Victor Romanov	Visiting Fellow	LMMB, NCI

COOPERATING UNITS (if any)
I. Tsarfaty, D.L. Faletto, G.F. Vande Woude, P. Sutrave, S. Rong, S.H. Hughes, J. Rasaw, I. Keydar, ABL-Basic Research Program, NCI-FCRDC; P. Sirigu, Dept. Anatomy, Univ. Cagliari School of Medicine, Cagliari, Italy; (continued on next page)

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Structural 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 ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Over the past year, work at the Structural Biology Section involved the use of a system of methods developed in our laboratory (in particular fracture-flip) and their combination with immunogold labeling. To this end we have continued and initiated new collaborative projects in areas of research where the contribution of our expertise and methods can be of immediate interest. Among other areas, current research involves: the immunogold localization of oncogenes and oncogene products (MET, Ski, MUC1); the study of the structure and topochemistry of the membrane skeleton (in activated platelets, as improved by deep-etching, in a hepatoma cell line); cellular and molecular mechanisms of endotoxin shock; nanoanatomy and immunogold labeling of vertebrate nervous tissues and GABA receptor transfected cells; maturation of the mouse mammary tumor virus (MMTV); nanoanatomy and topochemistry of the cell surfaces of protozoan parasites; immunogold fracture-flip cytochemistry of the laminin receptor protein.

Cooperating Units (Continued):

R. Anadon, I. Rodriguez-Moldes, Dept. Fundamental Biology, Univ. Santiago de Compostela, Spain; G. Puia, E. Slobodyansky, E. Costa, Fidia-Georgetown Institute for the Neurosciences, Univ. Georgetown, Washington,DC; Outside collaborators: C. Romero, M. Asunción Bosch, Dept. Biochemistry & Molecular Biology I, Universidad complutense de Madrid, Spain; L. Menéndez Arias, S. Oroszlan, Lab. Molecular Virology & Carcinogenesis, ABL-Basic Research Program, NCI-FCRDC; Collaborating People: P.F.P. Pimenta, Lab. Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, NIH; M. Edidin, Dept. Biology, The Johns Hopkins Univ., Baltimore, MD; Collaboration: T. Nash, D. Dwyer, Lab. Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, NIH; W. de Souza, W. Kattenbach, Lab. Parasitology, Univ. Rio de Janeiro, Brazil; D. Mirelman, The Weizman Institute of Science, Rehovot, Israel; V. Castronovo, M. Sobel, S. Aznevorian, L. Liotta, Lab. Pathology, National Cancer Institute, NIH.

PROJECT DESCRIPTION

Major Findings:

A. Immunogold localization of oncogenes and oncogene receptors (Shen, Sutrave, Zhou, Tsarfarty, Faletto, Rong, Stephen, Rasau, Hughes, Vande Woude, Pinto da Silva).

1. MET. The met protooncogene product (Met) and its ligand, hepatocyte growth factor/scatter factor (HGF/SF) have been implicated in cell mitogenic response, cell motility, and promotion of ordered spatial arrangement of tissue. The expression and activation of Met may be associated with epithelial cell differentiation to form lumen structures while reduced expression could be related to the extent of cell differentiation.

Our immunogold labeling shows that met protooncogene protein C28 is localized in the plasma membrane, cytoplasm and nucleus of met transfected NIH 3T3 cell lines (S6, S7) as well as T47D (a breast cancer cell line) and HT29 human colon cancer cell line. In T47D cells the met protein forms clusters inside high electron dense bodies inside the nucleus, transverse the nuclear envelope, the cytoplasm, and the plasma membrane, secreted, and concentrated on the microvilli of the lumen. HT29 cells, on the other hand, show a more restricted pattern of met protein distribution. Besides weak staining the nucleus, C28 accumulates inside the large secretory vacuoles that move towards the plasma membrane and burst into the lumen. [*Tsarfarty I, Rasau H, Shen RL, Pinto da Silva P, Faletto D, Vande Woude GF. The Met protooncogene receptor and lumen formation. Science. Submitted.*]

We will study the effect of HGF/SF and Met on lumen formation and embryo differentiation, their tumorigenic effect and tumor induction mechanism.

2. SKI. The oncogene ski was originally identified as the transforming principle in an avian retrovirus. Today, we know that the most intriguing aspect of ski is not its weak oncogenic potential, but its myogenic potential. Ski cannot only

induce myogenic differentiation in cultured quail cells, but can also induce muscle growth in transgenic mice (Sutrave et al. Genes Devel 1990;4:1462-1472).

Chicken embryo fibroblasts were infected with replication-competent retroviral vectors that express high levels of each of the three known chicken c-ski cDNAs. We have shown by immunofluorescence that when each of the three c-ski proteins is overexpressed it is localized primarily in the nucleus and, in metaphase cells, that c-ski protein associates with condensed chromatin (Sutrave et al. Mol Cell Biol, 1989;9:4046; Sutrave et al. Mol Cell Biol 1990;10:3137). These studies also revealed that the three forms of c-ski protein had different distribution within the interphase nucleus. The largest c-ski protein (FB29) has a punctate distribution as does FB28. The smallest form, FB27, is more uniformly distributed in the nucleus, but is clearly excluded from the nucleolus. We are investigating these differences in subnuclear localization by immunoelectron microscopy. The majority of the FB29 protein appears, in interphase cells, to be primarily localized in clearly demarcated particles we call "ski bodies" that are distinct from other organelles or structures. Some of the FB29 protein is associated with chromatin in interphase nuclei. FB28 protein appears to be almost exclusively localized within ski bodies, while FB27 protein is associated with chromatin. In FB29 expressing cells treated with nocadazole, ski bodies could be found throughout the cell. [*Shen RL, Pramod S, Pinto da Silva P, Vande Woude GF, Hughes H. Subcellular localization of ski oncogenes in chick embryo fibroblasts. Abstract for 5th International Congress on Cell Biology, Madrid, Spain, 1992.*]

We will now attempt to separate the ski bodies from the ski transfected cells to study the effect of ski on cell differentiation and transformation.

3. MUC1. MUC1 protein is a mucin type glycoprotein, over-expressed in breast cancer cells. Expression of MUC1 in eukaryotic cells diminishes the ability of these cells to attach to each other, resulting in single cell growth as opposed to a clump type of growth in the control cells. Elevated amounts of MUC1 are also detected in sera of metastic breast cancer patients people also show that MUC1 is located exclusively in the apical domain of the plasma membrane.

Using low temperature immunocytochemistry electron microscopy, replica labeling freeze-fracture and fracture-flip, we determined the subcellular localization, secretory pathway, and the polarity of MUC1 in breast carcinoma cell line T47D, NIH 3T3 cells transfected with genes coding for MUC1 (CA3, CA7, and CA8 subclone). We found that in MUC1 transfected NIH 3T3 cells, the MUC1 protein predominantly located on the plasma membrane and microvilli of the cells expressing MUC1 (about 50% in CA3, 25% in CA7 and CA8). Some MUC1 was detected inside the secretory vacuole. In the T47D cells, however, MUC1 was concentrated along the secretory vacuoles inside the cytoplasm, microvilli, junctional area, and junctional space. The concentration of the MUC1 is proportional to the width of the junctional space, indicating the effect of MUC1 in decreasing the cell-cell contact which is partially responsible for the metastatic behavior of tumor cells.

Two hypothesis exist on the secretion of MUC1: a) alternative splicing (splice out transmembrane domain before it is secreted--termination codon before the transmembrane region); b) posttranslational processing with cutting of the transmembrane portion of the molecule before it is secreted. Our results support the posttranslational processing model.

We will use monoclonal antibodies against different domains of MUC1 to investigate the secretion pathway of H23Ag.

B. The structure and topochemistry of the membrane skeleton and nuclear envelope membranes as seen by fracture-flip immunogold (Hou, Fujimoto, Aguas, Edidin, Pimenta, Mirmen, Kelly, Majuire, Pinto da Silva).

1. Ultrastructural differences between Xenopus egg nuclear pore complex and some selected somatic cell nuclear pore complex--a fracture-flip study. We confirmed the observations of Unwin for the organization of the nuclear pore complex in Xenopus egg. We found, however, that their validity cannot be extended to somatic cell nuclei (rat liver, human lymphoma cell line, human lymphoma cell line, human hepatocyte cell line) which, as seen by fracture-flip, lack the cytoplasmic ring first reported for Xenopus. Because fracture-flip methods developed in our laboratory can be applied to observe any isolated nucleus they are preferable to low dose microscopy examination followed by Fourier analysis as used by Unwin. Our results indicate that there are structural differences in the molecular organization of the nuclear pore complexes between germ cells and somatic cells.

2. The inner surface of platelet plasma membranes as revealed by fracture-flip and immunogold labeling. The cytoskeleton of platelets plays an important role in coagulation. Previous work (thin section, deep-etching) showed that stimulated platelets display membrane skeletons at the inner surface of the plasma membrane. We used fracture-flip followed by detergent extraction and ultrasonic vibration to reveal the inner surface of platelets. We found novel pebble-like structures on the inner surface of the membrane. In addition, we showed that actin is not connected to the cell membrane but, instead, is part of the cytoskeleton.

3. Fracture-flip followed by deep etching improves the resolution of images of membrane surfaces. In fracture-flip, carbon casts with their attached hemimembrane are air dried. We show that resolution of the images of membrane surfaces may be improved of, instead of air drying the carbon casts are re-frozen in liquid/solid N_2 and then etched in a Balzers apparatus. We conclude that air drying may lead to collapse of longer molecular chains exposed on membrane surfaces. Rapid-freezing followed by deep etching provides crisper, higher resolution images of both inner and outer surfaces of biological membranes.

4. The inner surface of plasma membrane in a hepatoma cell line as studied by fracture-flip immunogold labeling. MHC (major histocompatibility complex) contains both transmembrane molecules and nontransmembrane molecules. The movement of transmembrane molecules ($H\text{-}2D^b$) is thought to be hindered by a barrier ("corral"). This barrier appears to be sterical and to be effected by components of the membrane skeleton. In our experiments we used fracture-flip followed by detergent and protease treatments to reveal the inner surface of hepatoma cell membrane and to confirm the existence of the membrane skeleton networks. The size of net mesh spaces coincides with the distance available for movement of the transmembrane molecules (1 to 2 μ). The filament network was labeled with antispectrin colloidal gold.

C. Cellular and molecular mechanisms of endotoxin shock (Risco, Romero, Bosch, Pinto da Silva). Many effects of bacterial endotoxins on animals are a consequence of the overproduction of mediators by macrophages. The initial events that

activate macrophages have, therefore, a special priority in the study of endotoxic shock. We have studied the interaction of aggregated (usually employed in biochemical studies) and dispersed endotoxins (that we previously characterized at the ultrastructural level) with macrophages. By fracture-flip we have obtained high resolution images of the surface of these cells. Immunocytochemical studies allowed us to visualize the binding of the mentioned endotoxins to the macrophage membrane. Our results show the importance of using monodisperse preparations to study the physiological pathways of endotoxin action. The study of the interaction of endotoxins with other target cells is in progress. [*Risco C, Pinto da Silva P. Interaction of bacterial endotoxins with macrophages: influence of the state of aggregation of LPS. Infection and Immunity. Submitted.*]

D. Nanoanatomy and immunogold labeling of vertebrate nervous tissue and transfected cells (Caruncho, Anadon, Rodriguez-Moldes, Puia, Slobodyansky, Costa, Pinto da Silva).

1. Comparative morphology of teleost meninges. A project on the microanatomy of the biomembranes of teleost meninges and of their alterations during adaptations to darkness has been completed and two manuscripts submitted: [*Caruncho HJ, Pinto da Silva, P, Anadon R. The morphology of teleost meningocytes as revealed by freeze-fracture. J Comp Neurol. Submitted; Caruncho HJ, Pinto da Silva, P. Alterations in the intermediate layer of the goldfish meninges during adaptation to darkness. J Comp Neurol. Submitted*].

We found that teleost meninges are similar to mammalian leptomeninges but do not present a subarachnoid space filled with cerebrospinal fluid. One cell layer in the fish meninges shows well developed tight junctions that resemble the barrier layer of the mammal meninges. Fish (but not mammal) meninges also show gap junctions and desmosomes. Pinocytotic caveolae are more frequent in fish than in mammal capillaries; this and differences in the ultrastructure of the meningeal capillaries indicate that teleost meninges have a physiology distinct from that of the mammal meninges. We also demonstrated the role of the intermediate meningeal layer in an active process of transcytosis. We are now studying the ontogeny of the meninges in cyclostomes, elasmobranchs and teleosts.

2. Localization of native and recombinant GABAa receptors. We used immunogold labeling techniques to show the topochemistry of GABAa receptors with different subunit composition in cortical neurons, cerebellar granule cells and astrocytes. In addition, we analyzed the transient expression of the cDNA for the α, β, and τ subunits of the GABAa receptor transfected to transformed kidney cells. We found significant differences between the density of receptors in neurons, glial cells and transfected cells. Label-fracture and fracture-flip allowed us to show the presence of different subclasses of receptors in the same cell and to study their relative proportions. We complemented these studies with electrophysiological and pharmacological data. [*Caruncho HJ, Puia G, Slobodyansky E, Pinto da Silva P, Costa E. Expression of native and recombinant GABAa receptors as studied by freeze-fracture cytochemistry. In preparation.*]

E. Maturation of the mouse mammary tumor virus (MMTV) (Risco, Menéndez-Arias, Pinto da Silva, Oroszlan). Retrovirus maturation involves proteolytic processing of polyprotein precursors of viral proteins, in a step required to produce infective virions. Using EM immunocytochemistry we have complemented the

biochemical studies on the proteolytic activities associated with MMTV (Menéndez-Arias, Risco, Oroszlan, in press). The ultrastructural study of MMTV producing cell lines allowed us to establish adequate conditions that potentiate the cytoplasmic accumulation of immature viral particles, and to design a suitable method for their isolation. Immunocytochemical studies showed a radial distribution of the protein domains in intracytoplasmic A particles and provided an additional ground to explain its role in cell-capsid interactions such as the transport of A particles to the plasma membrane. [*Menéndez-Arias L, Risco C, Pinto da Silva P, Orozlan S. Purification of immature cores of mouse mammary tumor virus and immunolocation of protein domains. J Virol. Submitted.*]

F. Nanoanatomy and topochemistry of the cell surfaces of protozoan parasites (Pimenta, Kattenbach, Mirelman, Nash, Dwyer, de Sousa, Pinto da Silva). Freeze-fracture and fracture-flip of Giardia demonstrates distinct regions of the plasma membrane. Over the dorsal area we found projections and undulations that correspond to sites of endocytic activity, as well as vesicles lining the dorsal surface. These vesicles show positive phosphatase acid and they represent components of acidic compartments related with lysosomal complex responsible for the ingestion and accumulation of macromolecules by the parasite. [*Pimenta PFP, Pinto da Silva P, Nash T. Presence of thick cell coat in Giardia lamblia is associated with the variant surface antigens. J Cell Biol 1991;115(3 part 2):509-521.*]

We have now localized variant surface antigens on the cell coat of the organism. This coat was labeled with specific monoclonal antibodies and was not present in a distinct minority subpopulations. Parallel studies showed that the presence of distinct variant antigens are related with the parasite susceptibility to the intestinal enzymes trypsin and α-chymotrypsin.

In Leishmania, we showed that long-term culture of the parasites under specific conditions (pH and temperature) are differentiated in amastigotes (the form of the parasite that are able to develop inside the vertebrate host). The cell surfaces of cultivated amastigotes were similar of spleen-derived parasites. This may be of consequence in the study of the biochemical, antigenicity, and molecular changes associated with cell transformation. Leishmania parasites were also used to develop a method to visualize the inner surface of the plasma membrane. A modification of fracture-flip revealed the aspect of the cytoskeleton associated with the plasma membrane. High resolution tridimensional views associated with specific labeling confirmed the microtubular characteristic of this cytoskeleton. We also uncovered the structures that represent the cytoplasmic portion of proteins that link the microtubules with the plasma membrane.

Amoeba is another tropical parasite that we characterized with new ultrastructural and immunocytochemical approaches. Different strains of the parasites were associated with the pathogenicity according to distinct morphological aspects revealed by fracture-flip and freeze-fracture. Specific arrangements of integral proteins and typical filaments were only visible in infective pathogens. These results are important to characterize and differentiate different parasite strains.

G. Immunogold fracture-flip cytochemistry of the laminin receptor protein (Romanov, Castronovo, Sobel, Aznevorian, Liotta, Pinto da Silva). Laminin, a major basement membrane glycoprotein, plays an important role in a wide variety of

biological processes including tumor invasion and metastasis formation. Several membrane proteins have been described as laminin receptors, and their expression is markedly increased in highly metastatic cells. At present, while much is known on the molecular structure of these molecules, no data exist at the ultrastructural level on their topochemistry, i.e. their distribution on the cell surface. We investigate the distribution of these receptors and ultrastructural membrane alterations under different conditions. To this end, we use immunogold labeling methods as well as freeze-fracture (label-fracture, fracture-flip) and thin section (Lowicryl embedding) methods.

Publications:

Shen RL, Ward RD, Pinto da Silva P, Nishioka D. Localization of wheat germ agglutinin and mAb J18/2 binding sites in the plasma membranes of the sea urchin sperm as revealed by label-fracture and fracture-flip. Mol Reprod Dev 1991;28:410-418.

Shen RL, Aguas AP, Pinto da Silva P, Silva MT. Intramacrographic mycobacterium avium bacilli are coated by a multi lamellar structure: freeze-fracture analysis of infected mouse liver. Infect Immun 1991;59:3895-3902.

Renping Z, Shen RL, Pinto da Silva P, Vande Woude, GF. In vitro and in vivo characterization of $pp39^{mos}$ association with tubulin. Cell Growth and Diff 1991;2:215-266.

Doyle PS, Engel JC, Pimenta PFP, Pinto da Silva P, Dwyer DJ. Leishmania donovani: Long-term culture of axenic amastigotes at 37° C. Exp Parasitol 1991;73:326-334.

Pimenta PFP, Pinto da Silva P, Nash T. Variant surface antigens in Giardia lamblia are associated with the presence of a thick cell coat. A thin section and label-fracture immunocytochemistry survey. Infect Immun 1991;59:3895-3902.

Kattenback WM, Pimenta PFP, de Souza W, Pinto da Silva P. Giardia lamblia: A freeze-fracture, fracture-flip, and cytochemical study. Parasitol Res 1992. In press.

Sirigu P, Shen RL, Pinto da Silva P. Human meibomian glands: the ultrastructure of acinar cells as viewed by thin section and freeze-fracture transmission electron microscopies. Invest Ophthalmol Vis Sci 1992. In press.

Pavan A, Covelli E, Pascale MC, Lucania G, Bonatti S, Pinto da Silva P, Torrisi, MR. Dynamics of transmembrane proteins during Sindbis virus binding. J Cell Sci 1992. In press.

Fujimoto K, Pinto da Silva P. The cytoplasmic surface of human erythrocyte membranes as revealed by fracture-flip/Triton-X. Acta Histochem Cytochem 1992. In press.

Hou, WY, Pimenta PFP, Shen RL, Pinto da Silva P. Stereo-views and immunogold labeling of the pellicular microtubules at the inner surface of the plasma membrane of Leishmania as revealed by fracture-flip. J Histochem and Cytochem 1992. In press.

Menéndez-Arias L, Risco C, Oroszlan S. Isolation and characterization of α_2-macroglobulin-protease complexes from purified mouse mammary tumor virus and culture supernatants from virus-infected cell lines. J Biol Chem 1992. In press.

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

PROJECT NUMBER
Z01 CB 08389-05 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structure-Function Studies of Glycosyltransferases

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

Pradman K. Qasba, Ph.D.	Research Chemist	LMMB, NCI

Other Professional Personnel:

Arni Masibay Ph.D.	Staff Fellow	LMMB, NCI
Elizabeth Boeggeman Ph.D.	IRTA 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:
2.5	2.5	0.0

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

B

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

Glycosyltransferases have an inverted membrane topology that consists of short amino-terminal cytoplasmic tail, a hydrophobic anchor domain and the carboxyl-terminal portion of the protein that carries the catalytic domain. In the present studies we have identified a transmembrane region of the enzyme by transfection of Cos-7 cells with the amino-terminal deletions of beta-1-4galactosyltransferase cDNA. By exchanging the sequence segment of this enzyme with the corresponding region of other glycosyltransferases and using catalytic domain of 1-4galactosyltransferase as a reporter protein we have identified a transmembrane domain of alpha-1-3galactosyltransferase and of alpha-2-6sialyltransferase. These studies have shown that the amino-terminal signal anchor domain, the transmembrane region of glycosyltransferases is essential for the stability and targeting of these enzymes to the Golgi apparatus in mammalian cells. For the functional analysis of the carboxyl-terminal catalytic domain of beta-1-4galactosyltransferase the deletions and site directed mutants of the cDNAs were constructed in the pGEX vectors and expressed in E. coli. Either the full length or the amino-terminal deleted bovine galactosyltransferase were expressed as fusion proteins, connected to the COOH-terminus of glutathione S-transferase (GST), and purified on glutathione-affinity columns, cleaved by site-specific protease from GST, and each analyzed for GT and lactose synthetase (LS) enzymatic activities. Full length bovine GT (402 aa), and the proteins with deletions in the amino-terminal end up to residue 130 (GT 130) were active in GT and LS assays. Both GT and LS activities were lost when residues 130-142 were deleted. Within this deletion is the Cys 134 which when modified by site-specific mutagenesis to serine (GTc134s) or alanine (GTc134a) gives about 1% of the activity of GT 130. These results show that the Cys 134 which has been shown to be in a disulphide bridge with Cys 234 is necessary to impart the enzymatic function to the protein.

PROJECT DESCRIPTION

Objective:

To identify the sequence and/or structural motif of the transmembrane region of glycosyltransferases and its function in the biogenesis of the enzyme.

The protein sequences of glycosyltransferases, derived from the cDNA sequences, have implicated that these enzymes have inverted membrane topology that consists of short amino-terminal cytoplasmic tail, a hydrophobic transmembrane anchor domain and the carboxyl-terminal portion of the protein that carries the catalytic domain. The objective of the present studies is to determine by the recombinant DNA methodology: a) the sequence region of the trans-Golgi transferases that functions as the Golgi targeting sequence, and b) if there are common residues and/or a structural motif within this sequence that is being recognized during the targeting process.

To determine a) the maximum length of the "stem" region of beta-1-4galactosyltransferase protein that can be dispensed without effecting the catalytic function of the enzyme and b) to produce the protein in large quantities for the 3-D structure determination.

Analyses of the protein sequences of glycosyltransferases, derived from their cDNA sequences, have suggested that the catalytic domain of these enzymes lie in the carboxy-terminal portion of the protein. The objective of the present studies is to determine by the recombinant DNA methodology and by expressing the protein in E. coli: a) the length of the stem region that can be deleted and still retain the enzymatic function of the protein, b) identify the residues that are essential for the catalytic function of the protein, and c) to produce the protein in large quantities for 3-D structure determination.

Major Findings:

Preparation of deletion constructs of bovine beat-1-4galactosyltransferase cDNA in eukaryotic vector and their expression in COS-7 Cells:

A full-length bovine galctosyltransferase cDNA was constructed from a partial cDNA clone and a genomic fragment containing 5'-end sequences of the cDNA. The full-length cDNA was assembled on the Okayama-Berg vector where its expression is under the control of SV40 promotor. Upon transfection of Cos-7 cells with the resulting vector, pLsGT, there was about 12 fold increase in the galactosyltransferase activity. The results show that the bovine GT protein expressed transiently in COS-7 cells and coded by the cDNA is fully functional and that the binding sites for UDP-galactose, NAG, glucose and alpha-lactalbumin are all intact and operational.

Based on the general topological information of glycosyltransferases and the gene structure, we have generated many deletion constructs from the parent beta-1-4galactosyltransferase expression vector, pLsGT. We have deleted the DNA sequences which code for the potential membrane anchoring domain of the

galctosyltransferase protein and the first amino-terminal 70 residues which includes the "stem" region of the enzyme and is absent from the enzymatically active secreted form of the protein. Upon transfection of Cos-7 cells with these constructs no enzymatic activity could be detected although upon co-transfection with the parental construct or beta-galactosidase coding vector produced the specific proteins. The results suggest that the membrane-anchoring domain of beta-1-4galactosyltransferase has a role in mammalian cells either to stabilize the protein or to prevent it from inactivation.

Identification and Exchange of Transmembrane Regions of Glycosyltransferases:

Amino-terminal deletion mutants of beta-1-4galactosyltransferase which neither exhibted any enzymatic activity nor produced any protein upon transfection of Cos-7 cells, were fused with the sequences which coded for the putitative transmembrane domains of alpha-1-3-galactosyltransferase and alpha-2-6-sialyltransferase. Ovelapping 5'-end sequences, representing the amino-terminal portion of these proteins, were synthesized and assembled into a DNA segment that was fused with the deletion mutants of beta-1-4galactosyltransferase. In this method catalytic domain of beta-1-4galactosyltransferase protein acted as a reporter protein and its production and activity was rescued by the sequences attached at the amino-terminal end that targetted the protein to the Golgi compartment. By this method we identified sequences which represent the amino-terminal anchor sequences or transmembrane regions of these glycosyltransferases.

Cellular Localization of the Expressed Protein in the Transfected Cells:

We have used a monospecific polyclonal antibody against highly purified bovine beta-1-4galactosyltransferase to identify and localize the expressed protein in the permeabilized and non-permeabilized transfected cells using a florescence double-antibody technique. These results have shown that galactosyltransferase with either beta-1-4- or alpha-1-3 transmembrane domain is localized in the Golgi compartment.

Preparation of deletion constructs of bovine beta-1-4galactosyltransferase cDNA in prokaryotic vector and their expression in E. coli:

Bovine beta-1-4galactosyltransferase was expressed in E. coli as a fusion protein connected at the carboxy-terminal end of glutathione S-transferase using the novel pGEX expression system which allows very high yield of recombinant proteins after a single-step purification. The pGEX vectors contain the carboxyl terminus of the glutathione S-transferase gene from Schistosoma japonicum under the control of a tac promoter. Full-length cDNA was inserted into the multicloning site of the vector pGEX to achieve the production of the fusion protein with the glutathione S-transferase as an affinity tail in the correct orientation and open reading frame. After ligation and transformation of E. coli a clone was identified that contained the plasmid with the insert in right orientation and restriction sites. After induction with IPTG E. coli cells were sedimented, lysed and pelleted. The

supernatant was applied to glutathione-sepharose column. The fusion protein that sedimented with the pellet was denatured with 6M guanidium-Hcl, renatured by dialysis and purifed on an affinity coloumn. The bound fusion protein was cleaved with factor X.

A cDNA sequence, which would code for the secreted form of the protein that lacks the amino-terminal 70 residues, was isolated from the full-length cDNA by Sst I and Eco RI restriction digestion. The DNA fragment was first subcloned into pSPT18 vector and then engineered into pGEX-2T vector to achieve the production of the fusion protein which was cleaved with thrombin to release the secreted form of beta-1-4galactosyltransferase. Further amino-terminal deletion constructs were generated by PCR amplification of the desired regions of the cDNA with the 5' and 3' end primers that carried Bam HI and Eco RI restriction sequence, respectively, for the site directed insertion in the pGEX2T vector.

Full length bovine GT (402 aa), and the proteins with deletions in the amino-terminal end up to residue 130 (GTD130) were active in galactosyltransferase (GT) and lactose synthetase (LS) assays. Their activities increased as the amino-terminal residues were progressively trimmed from 1 to 130 which include the short cytoplasmic tail, the signal-anchor domain and the stem region of the protein. This is in contrast to the results observed in mammalian cells where the secreted form of the protein within the cell is not active, for as yet unknown reasons. However, both GT and LS activities are lost when residues 130-142 are deleted from the protein produced in E. coli, which includes the Cys 134 that has been shown to be joined in a disulfide bond with Cys 247. When GT 130 cDNA was modified by site-specific mutagenesis to replace Cys 134 by serine (GTc134s) or alanine (GTc134a) the resultant mutants have about 1% of the activity of GT 130. These results show that the Cys 134 which has been shown to be in a disulphide bridge with Cys 247 is necessary to impart the enzymatic function to the protein. For the 3-D structure determination large quantities of these protein are being purified.

Publications:

Masibay AS, Damewood GP, Boeggeman E, Qasba PK. Regulation of beta-1-4galactosyltransferase during cell growth. Biochimica et Biophysica Acta 1991:1090;230-234.

Masibay AS, Boeggeman E, Qasba PK. Deletion analysis of the amino-terminal region of the beta-1-4galactosyltransferase protein. Mol Biol Rep 1992:16;99-104.

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

PROJECT NUMBER
Z01 CB 08392-04 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Combination Therapy of Cancer and AIDS

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

John N. Weinstein, M.D., Ph.D., Chief, Theoretical Immunology Section LMMB, NCI

Other Professional Personnel:

Vellarkad N. Viswanadhan, Ph.D.	Visiting Scientist	LMMB, NCI
Hitoshi Sato, Ph.D.	Visiting Fellow	LMMB, NCI
Miklos Peterfy, Ph.D.	Visiting Fellow	LMMB, NCI

COOPERATING UNITS (if any)
LTCB, DCBDC, NCI; LMI, NIDR; O.D., DCE, NCI; Dept of Pathology, Uniformed Services Univ. of Health Sci.; P.R.I., NCI/FCRDC; Clin. Pharm. Dept., Johns Hopkins U. Med. School; AIDS Unit, Henry Jackson Foundation

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Theoretical Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0.0

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 developed a new "modulatory" combination therapy for AIDS. Dipyridamole (DPM; Persantin), a potent inhibitor of nucleoside transport, is widely used for cardiovascular indications. We found that DPM potentiates the activity of azidothymidine (AZT) against human immunodeficiency virus (HIV-1) in cultured human monocyte/macrophages and stimulated T-cells. In cultured human T-lymphoblastoid cells, DPM potentiates the antiviral activity and simultaneously protects the cells from AZT's cytotoxicity. DPM does not potentiate AZT's cytotoxic effect on human bone marrow progenitor cells in vitro. Taken together, these findings suggest that DPM may increase the therapeutic index of AZT in vivo. We are currently collaborating with two other institutions on clinical trials of the AZT/DPM combination. Other aspects under study include:

1. Mechanism: DPM blocks cellular uptake of physiological nucleosides but not of AZT. The potentiation of AZT may thus result, in part, from decreased influx of the nucleosides that compete with AZT for viral reverse transcriptase.
2. Molecular structure: A structure for DPM has been computed from the crystallography. Quantitative structure-activity relationships (3D-QSAR) are being studied to predict which features of nucleoside transport-inhibiting molecules are required for activity.
3. Molecular biology: Methods based on polymerase chain reaction are being used to clone the nucleoside transport protein, a major target for DPM.
4. Analysis of combination therapy: Because no published algorithm or computer package was adequate for analysis of our data on antiviral drug combinations, we have developed a new approach. The new concepts and prototype computer program package (COMBO), will be useful in the context of cancer as well as AIDS.

PROJECT DESCRIPTION

Major Findings:

1. Under some conditions of culture, dipyridamole (DPM) inhibits replication of HIV-1 in cultured human monocyte/macrophages; more strikingly, it potentiates the anti-HIV activity of AZT and other dideoxynucleosides in those cells.

2. DPM potentiates AZT against HIV-1 in human T-lymphocytes (i.e. phytohemagglutinin-stimulated, IL2-propagated mononuclear cells).

3. DPM potentiates AZT against HIV-1 in a T-lymphoblastoid cell line (CEM-SS) and simultaneously protects those cells against the cytotoxic effects of AZT. Thus, the "in vitro therapeutic index" for those cells is greatly increased.

4. DPM does not potentiate the toxicity of AZT for human bone marrow progenitor cells in a CFU GM assay.

5. DPM inhibits uptake of thymidine by monocyte/macrophages, whereas it does not inhibit uptake of AZT. This "differential transport inhibition" may be one of the mechanisms underlying the potentiation of AZT activity, but other effects are probably operating as well.

6. In addition to its effect on transport, DPM appears to inhibit phosphorylation of the thymidine directly. This effect may also contribute to the antiviral activity.

7. DPM has been reported to induce interferon, but we find no such induction in our studies. However, we do find (by a combination of bioassay and polymerase chain reaction studies) that HIV-infected monocyte/macrophage cultures produce alpha-interferon, at least part of which is acid-labile. This interferon may give rise to some of the debilitating symptoms of AIDS, and it has been reported as an early predictor of the onset of clinical disease in infected individuals.

8. We find by a combination of ultrafiltration and equilibrium dialysis studies that DPM does not bind as strongly to serum proteins as has been suggested by less extensive studies in other laboratories. This finding is favorable with respect to the possibility of achieving clinically effective concentrations in vivo.

9. Three-dimensional quantitative structure activity relationship (3D-QSAR) studies yielded predictions with regard to physical chemical and geometric characteristics of the binding site of the nucleoside transport protein. The most robust predictions are: 1) that the anti- conformation of the ligand is preferred; 2) that the 5'-OH group of the ligand hydrogen-bonds to the binding site cavity of the transporter. These predictions are useful in the search for nucleoside analogues that interact effectively with the transporter.

10. A new set of concepts and algorithms has been developed (in collaboration with Dr. B. Bunow, Civilized Software, Inc.) for analysis of data on drug synergy and antagonism. The approach starts with a series of expressions based on enzyme kinetic models and heuristic principles. A program package called "COMBO" was developed to perform the following tasks, operating in the MLAB computing environment: (i) globally fit each model by least squares regression with constant weights; (ii) use the results in a Gaussian-windowed kernel re-weighing technique to generate updated weights; (iii) repeat regression with the updated weights; (iv) iterate the previous two steps; (v) use a combination of normal theory and Monte Carlo techniques to determine confidence limits on the fitting parameters; (vi) construct a set of derived parameters that express various aspects of synergy, potentiation, and antagonism; (vii) calculate confidence limits on those constructed parameters; (vii) produce graphical displays of the error model, the residuals of each fit, and the contours of equipotent drug effect. The new conceptual framework and prototype computer program package have been applied to the design and analysis of experiments on anti-HIV agents including AZT, ddC, ddI, ddA, dipyridamole, interferons, tumor necrosis factor, suramin, CD4-pseudomonas exotoxin, and protease inhibitors, *inter alia*. In the context of cancer, we have analyzed combinations including doxorubicin, suramin, tumor necrosis factor, dipyridamole, interferons, and dideoxynucleosides.

11. A pharmacologically-oriented clinical trial of DPM/AZT has been completed (a collaboration with Dr. Craig Hendrix (Henry Jackson Foundation) and with the group of Dr. Paul Lietman (Johns Hopkins University). The results are being evaluated.

AIDS Research:

This entire project is directly related to AIDS research (although the work on DPM, on nucleoside transport and metabolism, on cloning of the transporter protein, and on analysis of combination therapy (COMBO) clearly applies to cancer as well).

Bibliography:

Patel SS, Szebeni J, Wahl LM, Weinstein JN. Effect of dipyridamole on transport and phosphorylation of dideoxycytidine and cytidine in human monocyte-macrophages. Biochem Parmacol 1991;35:1250-1253.

Szebeni J, Dieffenbach C, Wahl SM, Venkateshan CN, Yeh A, Popovic M, Gartner S, Wahl LM, Peterfy M, Friedman RM, Weinstein JN. Induction of interferon-alpha by human immunodeficiency virus type-1 in human monocyte-macrophage cultures. J Virol 1991;65:6362-6364.

Szebeni J, Weinstein JN. Dipyridamole binding to proteins in human plasma and in tissue culture media. J Lab Clin Med 1991;117:485-492.

Weinstein JN, Bunow B. COMBO: New concepts and methods for designing and analyzing experiments on combination therapy. In: Goldestein AL, Garaci E, eds. Combination Therapies: Biological response modifies in the treatment of cancer and infectious diseases. New York, New York:Plunum Press 1992:29-38.

Kageyama S, Weinstein JN, Shirasaka T, Kempf DJ, Norbeck DW, Plattner JJ, Erickson J, Mistuya H. In vitro inhibition of HIV-1 replication by c2 symmetry-based HIV protease inhibitors as single agents or in combination. Antimicr Agents Chemother, in press.

Patents:

Chemotherapeutic composition for AIDS. Application plus continuation in part filed.

New antiretroviral agents and delivery system for the same. Application plus continuation in part filed.

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

PROJECT NUMBER
Z01 CB 08394-04 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Non-Contiguous Patterns and Functional Domains in DNA

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

Andreas K. Konopka, Ph.D.	Visiting Scientist	LMMB, NCI
Other Personnel:		
John Owens	Computer Specialist	LMMB, NCI
Joanne Austin	Student Intern	LMMB, NCI

COOPERATING UNITS (if any)
Dr. Danielle Konings, Univ. Colorado, Boulder, CO; Dr. Hugo Martinez, UCSF, San Francisco, CA; Dr. Pavel Pevzner, USC, Los Angeles, CA; Dr. Peter Salamon, SDSU, San Diego, CA.

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.75	1.0	0.75

CHECK APPROPRIATE BOX(ES)
☐ (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.)

Goals of this project are:

1) Develop theoretical foundations for sequence analysis research.

2) Provide a consistent methodology for locating putative functional domains in unannotated nucleic acid sequences.

Computational experiments have been performed to investigate which of the existing methods (including those resulting from the previous years of this project) gives the best estimate of the location of distinct functional domains in unannotated nucleotide sequences. It has also been necessary to examine the very theoretical foundations of sequence analysis. In particular, conceptual algorithms enabling one to interpret nucleotide sequences through a linguistic framework have been developed and implemented in new sequence analysis software. Universal information-theoretic principles governing distribution of short oligonucleotides have been discovered and are now being studied in detail. It is believed that the novel principles provide a powerful basis for discriminant analysis algorithms and, on the other hand, might shed some light on the mechanistic aspects of genome fragments' (not only genes') expression.

An international workshop (Open Problems of Computational Molecular Biology, Telluride, CO, June, 1991) has been organized to address the so called biological coding problem emerging from this project. The formulation of this problem and methods for its solution are now being studied in several research groups worldwide.

PROJECT DESCRIPTION

Major Findings:

1. BACKGROUND, RATIONALE AND TERMINOLOGY.

Molecular Biology came to life at a time when scientific methodology was dominated by linguistic philosophy and major breakthroughs in logic. Several fields in cognitive science were also born at this time, including "information" theory, formal linguistics and computer science. No doubt, the scientific community of this period was well prepared to discuss their methods and results within a metaphoric context of language, communication and computation. For biologists the genome was no longer an abstract pool of genotypes in a population of a species, but the complete DNA of an organism. Each strand of a double-stranded DNA molecule turned out to be a linear copolymer of four monomers (nucleotides). In addition, if the nucleotide sequence of only one strand is known, simple application of the Watson-Crick base pairing rules allows us to restore the sequence of the other strand. Therefore any DNA molecule can be represented as a linear string of four symbols, exactly like a written text that is a linear string of symbols from a finite alphabet. The psychological consequence of this text metaphor was a presupposition that DNA can be a carrier of "messages". The spectacular discovery of the translation code for proteins and messenger RNA reassured biologists that this presupposition might indeed have been correct. Therefore the genome could be regarded as a collection of messages written in one or more unknown languages.

There are limitations for such a loosely formulated language (or text) metaphor. If the genome were indeed a "text" it would be, in the first place, an encoded text. Not only might regions corresponding to different biological functions correspond to different "languages" but they might also be encoded according to different principles (so to speak the "cryptosystems" may be different in addition to language differences).

Moreover, the genome is not a static entity but undergoes changes during a cell's lifetime. Recombinations in which DNA can change include among other possibilities: unequal crossing over, region conversion, transposition, retrotransposition and integration of foreign (for example viral) DNA. Because of this dynamic nature of the genome, we need to take into account the possibility of multiple functions superimposed in the same DNA sequence.

Finally, even clearly functional regions in the genome are only occasionally expressed. For instance, in a specialized somatic cell, the genome carries several thousand protein coding genes. All of those genes have some potential to be expressed, but only a few of them are indeed transcribed and even fewer are translated to polypeptides. The temporal order of molecular events is undoubtedly critical during cell development. It is currently unknown how the "program" for this order could be encoded in the genome and it seems unlikely that the language metaphor alone will lead to an answer. From a logical standpoint sentences need to be described by meta-sentences,

meta-sentences by meta-meta-sentences and generally a formal system (a language) requires a corresponding meta-system (a meta-language) for its description. By analog, a clearly functional region in a genome (like a protein encoding gene) is subject to multiple levels of regulation that control the time and extent of its expression. Most sequences that are deposited in databases are obtained under conditions in which they are indeed expressed. Therefore, those sequences reflect not only the desired function but also the regulatory requirements from an unknown level of regulation.

We do not know what structural patterns observed in the genome reflect the actual "language" and what patterns are caused by the nature of "encoding" or "decoding" the alleged function in the presence of the above mentioned limitations. What we can know is the occurrence of selected structural patterns in (and the alleged function of) genome fragments. Based on such a knowledge we could construct classification codes that would represent (predefined) functions as sets of patterns (i.e. code words). In this setting the language metaphor can no longer refer to the mass-media-advocated "language of life", "genomic codes" or "genetic script". At best it could refer to our own classification codes (i.e. our own meta-languages) and the process of their construction.

2. RESULTS

1) A working classification of sequence (or structure) patterns has been proposed. The two main categories are contiguous and non-contiguous patterns. Each of these classes contains extendable and non-extendable pattern. Accordingly the four kinds of patterns (extendable non-contiguous, extendable contiguous, non-extendable non-contiguous, non-extendable contiguous) can be considered in any long string of symbols over a finite alphabet.

2) A general theory of discriminant analysis has been developed and implemented on all sequence data (35 large collections of functionally equivalent sequences) available through the GenBank. The theory is based on the discovery that the distribution of local compositional complexity is as random as possible consistent with the mean value of complexity. This novel maximum entropy principle provides a powerful theoretical tool to distinguish between the purely statistical effects and the biological constraints in nucleotide sequences. On the other hand the slope values of linear regression function (relating complexity with frequency of occurrence of short oligonucleotides) is a very sensitive indicator of whether a given sequence potentially belongs to a given class of functionally equivalent sequences. The discriminant analysis software based on this indicator is now being developed.

3) The biological coding problem has been formulated and methods for its solution have been examined. The most general version of the problem is: Given several (known) classification codes determined in a given unannotated sequence, choose the code that corresponds best to an (unknown) functional code. Among the methods of selecting "best" classification codes the following three seem to be the most promising: word length tests based on indices of coincidence, lambda test based on statistics of absent patterns and

complexity divergence index based on the distribution of local compositional complexity.

3. SIGNIFICANCE:

Reliable domain mapping programs will be indispensable for understanding data that emerge from various genome sequencing projects. Before such reliable programs will be designed we have to understand the principles of evaluating functional significance of sequences without actually knowing the function.

4. PUBLICATIONS:

Konopka AK. Sequences, codes and functions. Computers and Chemistry, 16, in press.

Salamon P, Konopka AK. A maximum entropy principle for the distribution of local complexity in naturally occurring nucleotide sequences. Computers and Chemistry, 16, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08396-04 LMMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Information Theory in Molecular Biology

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

Thomas D. Schneider, Ph.D.	Senior Staff Fellow	LMMB, NCI
Other Personnel:		
Denise Rubens	Research Associate	PRI/FCRDC
Paul N. Hengen, Ph.D.	Staff Fellow	LMMB, NCI
R. Michael Stephens	MIT, Cambridge MA; SRTP	LMMB, NCI

(continued)

COOPERATING UNITS (if any)

Dhruba K. Chattoraj and Peter P. Papp, Lab. of Biochemistry, NCI (Bethesda, MD); David Draper, Johns Hopkins (Baltimore, MD); Peter K. Rogan, Div. of Genetics, Univ. of PA (Hershey, PA); Kenneth E. Rudd, NLM, NIH (Bethesda, MD); (continued)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.50	1.5	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.)

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 usually contain just about the amount of information needed 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 in 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. Last year 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. Publications were completed at all three levels.

Principal Investigators (Continued)

Mark C. Shaner	SRTP	LMMB, NCI
Ian Blair	SRTP	LMMB, NCI
Stacy L. Bartram	SIP	LMMB, NCI

Cooperating Units (Continued):

Stanley Brown, ABL, Mol. Control and Genetics (Frederick, MD); Sharlene R. Matten, Dept. of Biochemistry, Univ. of MD (College Park, MD) and William S. A. Brusilow, Wayne St. Univ. (Detroit, MI); John Spouge, National Center for Biotechnology Information, NLM (Bethesda, MD); Peter Basser, DRS, BEIB (Bethesda, MD); John Garavelli, PIR, National Biomedical Research Foundation (Washington, DC); Nathan Herman, Frederick H.S. (Frederick, MD) and US Coast Guard Academy (New London, CT).

PROJECT DESCRIPTION

Major Findings:

Molecular Machines

I presented the theory of molecular machines as a poster in the Second Foresight Conference on Molecular Nanotechnology (Palo Alto, CA, November 7-9, 1991). It was well received. A review of molecular machine theory (levels 0 through 2) was written for the proceedings of this meeting and the journal Nanotechnology (Schneider, 1992).

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., 1992) 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.

Two Fold Excess Information Content: T7 Project

The goal of this project is to determine by experimental methods the fine structure of bacteriophage T7 promoters, because the sequences at these promoters are more conserved than is necessary for them to be found. Denise Rubens discovered that the plasmids into which variant T7 promoters were

cloned are at a lower copy number than required for ABI non-radioactive sequencing. We therefore switched to a PCR sequencing technique, months before ABI released kits to do this. Denise then found that standard primers did not work. When new primers were designed and synthesized, she succeeded in obtaining T7 promoter sequences by PCR amplification from single colonies. She is now sequencing variant T7 promoter sequences. We are planning to use the new magnetic bead DNA isolation procedure to increase the rate we obtain sequences. This will put us at the forefront of DNA sequencing technology. We will be sequencing at least 1000 clones, with the aim of understanding the three-dimensional structure of sequence contacts made by the T7 RNA polymerase. The sequence logo technique is giving us clues about what to expect and look for in this project.

Three Fold Excess Information Content: F incD Project

Last year's discovery of the three-fold information excess in the F plasmid incD region is now described in a small paper in press (Herman and Schneider, 1992). Paul Hengen, who joined my lab in April as a postdoc, has begun experimental work to dissect this system. Our direct approach, using methods developed in this lab, should reveal the DNA binding components required for the precise partitioning of newly replicated DNAs into daughter cells. Paul will simultaneously be looking at eukaryotic centromeres using information theory techniques.

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 (NIH, Bethesda), we have synthesized many variations of the RepA binding site. We then selected, cloned and sequenced 97 of those that still bind to RepA. As predicted, the anomalous peak was absent from the experimental sequence logo. 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. Information analysis and experimental work revealed even more anomalies in the binding sites. We now have evidence that the sites may be bound by a second protein, and that they contain a non-B DNA distortion. Our paper (in preparation) has been expanded to describe these new results, and to incorporate the information analysis of several other genetic control systems, including yeast GCN4 and GAL4.

Eukaryotic Enhancer Project

The yeast protein GCN4 binds to enhancer sites to stimulate transcription. My high school students 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. These results will probably be described in the paper on RepA. Mark 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 the data he produced to demonstrate this fit together within 2%! We plan to write a paper this summer on these results. For his work, Mark placed into the top 300 of the Westinghouse Science Talent Search, won top honors in the Frederick county science fair, and is at the International Science and Engineering Fair as of this writing.

Surprisingly, every student in the NCI Student Intern Program (SIP), of which Mark is the last, has made major discoveries which lead to important papers. E. coli Database Project Kenn Rudd of the National Library of Medicine has collected all known E. coli DNA sequences as a continuous clean data set representing 40% 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 Kenn, I have analyzed over 1000 E. coli ribosome binding sites (Rudd and Schneider, 1992). This data set is nearly 10 fold larger than the original analysis done in about 1984, and thus offers a refined look at the structure of the sites. A surprising, but tentative, result is that the information content of the binding sites is not sufficient for them to be located in the genome. Several explanations are possible, ranging from data problems to a spectacular mathematical proof that ribosomes scan the RNA linearly. We plan to investigate this new anomaly in detail. My new SIP high school student, Stacy Bartram, will probably be working with Kenn and me to analyze many other binding sites in E. coli.

Ribosomal RNA Project

In collaboration with David Draper of 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.

DNA Sequencing Project

A method concerning DNA sequencing is under an active patent search.

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.

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 was presented at the American Society for Microbiology in Dallas, Texas.

Automated Beta Galactosidase Assay

Many years ago Gary Stormo (University of Colorado, Boulder) and I wrote programs to gather and analyze data from beta-galactosidase assays done in 96 well plates. The method was recently improved by Dennis N. Arvidson and Philip Youderian (California Institute of Biological Research, La Jolla, CA) and finally published with us (Arvidson, et al., 1991).

Splice Junction Analysis

A paper with R. Michael Stephens, described in last year's report, was submitted for review. It is now in revision for the Journal of Molecular Biology.

Multiple Sequence Alignment

Michael Stephens is continuing to develop an information theory based multiple alignment program.

International Electronic News Group

John Spouge, John Garavelli and I found that it was difficult to maintain our informal electronic mail discussions as new people joined our group. In the beginning of this year we created a world-wide electronic news group (bionet.info-theory) for discussions of the use of information theory in biology. Discussions on the group have been quite lively since then.

AIDS research

1% (2 days) Discussions on the electronic net showed that people were having a difficult time deciding where to place PCR primers on the HIV genome. The phylogenetic technique (Rogan et al., 1992) suggested that a sequence logo of the entire genome would be useful for quantitatively identifying conserved

variable regions. We may use Mike Stephen's program to do the multiple alignment.

Publications:

Arvidson DN, Youderian P, Schneider TD, Stormo GD. Automated kinetic assay of beta-galactosidase activity. Biotechniques 1991;11:733-738.

Herman ND, Schneider TD. High information conservation implies that at least three proteins bind independently to F plasmid incD repeats. J Bacteriol 1992, in press.

Rogan PK, Salvo JJ, Stephens RM, Schneider TD. Visual display of sequence conservation as an aid to taxonomic classification using PCR amplification. In: Pickover C, ed. The Visual Display of Biological Information, 1993, and Speculations in Science and Technology. Teaneck, New Jersey:World Scientific 1993, in press.

Rudd KE, Schneider TD. Compilation of E. coli ribosome binding sites. In: Miller J, ed. A short course in bacterial genetics: A laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1992, in press.

Schneider, TD. Genetic patterns as shown by sequence logos. In: Pickover C, ed. Pattern Book. Teaneck, New Jersey:World Scientific, 1993, in press.

Schneider TD. Protein patterns as shown by sequence logos. In: Keller PR, ed. Visual Recipes: A Scientist's Guide to Visualization, 1993, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08397-01 LMMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Computer Modelling of Sugar Binding to Alpha-Lactalbumin.

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

Pradman K. Qasba, Ph.D.	Research Chemist	LMMB, NCI

Other Professional Personnel:

Petety Balaji, Ph.D.	Visiting Fellow	LMMB, NCI
Vallurupalli S. R. Rao, Ph. D.	Visiting Scientist	LMMB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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 amino acid requirements for binding of Ca2+ in alpha-lactalbumin have been studied. Comparison of the sequences of Ca2+-binding region of alpha-lactalbumin with the corresponding regions in the Ca2+ and non-Ca2+ c-type lysozymes suggest that in addition to the residues which have been identified by the X-ray crystal structure analysis as liganded to Ca2+ there are neighboring residues which also contribute to the binding of calcium ion.

Computer modelling methods are being used to address the question "why alpha-lactalbumin does not bind any sugar while as its homologous protein c-type lysozyme with which it has both sequence and structural homology does bind and hydrolyse oligosaccharides"? The three dimensional structure of alpha-lactalbumin and lysozyme are very similar but the two proteins have different functions. Lysozyme catalyses the hydrolysis of a beta(1-4)glycosidic linkage in polysaccharides, while as alpha-lactalbumin does not bind sugar by itself but does interact with the enzyme beta1-4galactosyltransferase, modifying its substrate specificity in a way which promotes the transfer of galactose to glucose resulting in a beta(1-4) linkage. Using computer modelling methods we have identified the side chain of amino acid residues in alpha-lactalbumin that block the entry of mono-saccharides into the C, B and D sites, equivalent to the one identified as sugar binding sites in the lysozyme. The side chain alterations in the protein that will allow binding of the sugar have been predicted and are being tested by the site directed mutagenesis of alpha-lactalbumin.

PROJECT DESCRIPTION

Objective:

1) To compare the calcium binding loop of alpha-lactalbumin with the corresponding region of c-type lysozyme.

2) To identify by molecular modelling methods the residues and any alterations in the alpha-C atom backbone of alpha-lactalbumin which blocks the binding of sugar moiety into the site equivalent to that of sugar binding site of structurally homologus protein lysozyme.

Although the 3-D structures of alpha-lactalbumin and c-type lysozyme are very similar they are functionally different. Alpha-lactalbumin is a metalloprotein that binds calcium and does not bind any sugar even though it does show structural similarity with the A, B, C, D, E, & F sugar binding sites of lysozyme. Lysozyme has a loop structure similar to the calcium binding loop structure of alpha-lactalbumin. Lysozyme binds a hexasaccharide, (-NAM-NAG-)3, in the A to F sites, where D-E is the catalytic site. The objective of the present molecular modelling work is to identify the differences in these sites which block the binding of mono- or di-saccharides and to compare and identify the dissimilarities in the loop region of the two proteins.

Major Findings:

The amino acid requirements for the binding of Ca2+ in alpha-lactalbumin:

Alpha-lactalbumin, a metalloprotein that binds Ca2+, modifies the enzymatic activity of beta(1-4)galactosyltransferase in a way that it promotes the transfer of galactose to glucose to produce lactose. It is evolutionarily related to c-type lysozyme that catalyses the hydrolysis of a beta1-4glycosidic linkage in polysaccharides. Lysozymes from different species, with the exception of horse, dog and pigeon, do not bind Ca2+. The 3-D structure of these two homologous proteins, alpha-lactalbumin and lysozyme, as well as their gene structures, are very similar. The alpha-carbon atom backbone of the Ca2+-binding loop of alpha-lactalbumin is very similar to the corresponding region of c-lysozymes but different from the EF-structure of other Ca2+-binding proteins. The high resolution X-ray structure analysis of baboon alpha-lactalbumin shows that Ca2+-binding site is located in a bend or elbow formed from 10-residues. Five of these are liganded to Ca2+ ion. Of these three are aspartic acids that interact with the bound Ca2+ ion through their side chain carboxyls. The remaining two residues interact through their carbonyl group, one of them being lysine. Oxygen of two water molecules is also coordinated with calcium giving rise to a distorted pentagonal bipyramid configuration. The sequence comparison of this region among all alpha-lactalbumins and Ca2+-binding lysozymes shows that the lysine residue (with the exception of one which has asparagine instead of lysine) along with aspartyls have been conserved at the corresponding positions within these proteins. Also within the loop two hydrophobic residues at corresponding positions have been conserved among alpha-lactalbumins and Ca2+-binding lysozymes. Looking at the

conserved among alpha-lactalbumins and Ca2+-binding lysozymes. Looking at the interatomic distances between various groups in this region the side chain amino group of the conserved lysine (or Asn) may be interacting with the water molecule coordinated with Ca2+ via another water molecule. Also the conserved hydrophobic residues may be involved in controlling the hydration sphere of the bound Ca2+ ion which results in more stable cation complex. Genetic manuplation of the alpha-lactalbumin cDNA clone is directed towards testing this hypothesis.

Identification of amino acid residues that block sugar binding in alpha-lactalbumin:

Computer modelling approach has been used to study the binding of monosaccharides to C, B and D sites of alpha-lactalbumin and lysozyme. Based on the contact criteria and energy parameters appropriate for monosaccharides, and using a systematic docking procedure - translations and rotations - the right fit binding of NAG has been studied. These molecular modelling methods suggest that Tyr 103 may block the entry of sugar into the C site of alpha-lactalbumin. Mutation of Tyr 103 to Ala 103 would allow binding of the sugar in the C site with a flipped orientation of the monosaccharide. The B site of alpha-lactalbumin is closed for NAM entrance relative to the B site of lysozyme due to the deletions and insertions in the neighboring region of the protein. Using these modelling methods for binding of NAG and NAM to the C and B sites, respectively, of the lysozyme molecule the hydrogen bonding schemes have been determined which are in agreement with the crystallographic structure of the lysozyme-sugar complex.

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

PROJECT NUMBER
Z01 CB 08398-01 LMMB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Uses of Artificial Intelligence to Optimize the Cancer Drug Development Process

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

John N. Weinstein, M.D., Ph.D.	Chief, Theoretical Immunology Section	LMMB, NCI
Hitoshi Sato, Ph.D.	Visiting Fellow	LMMB, NCI

COOPERATING UNITS (if any)
In the Developmental Therapeutics Program of DCT: ITB, LMP, LMC, DSCB. In the Clinical Oncology Program of DCT: MB, CPB; In DCRT: LAS, CSL.

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Theoretical Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	0.0

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

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

Artificial intelligence methods and classical statistics are being applied, in combination, to the task of analyzing and optimizing the cancer drug development pipeline. The Anti-Cancer Agent Predictions Working Group (ACAP) has been formed to coordinate these efforts. Initial results are as follows:

(1) Development of neural networks (MecNet) capable of predicting mechanism of drug action on the basis of patterns of activity in the 60-cell line cancer drug screen;

(2) Development of a program (DISCOVER) for identification of new structural and functional motifs among the tens of thousands of agents tested in the screen;

(3) Use of clustering in combination with neural networks and discriminant analysis to identify candidate cell lines for replacement in the screen (by breast, prostate, target-selected, target-transfected, and non-malignant lines, as appropriate).

(4) Preliminary predictions of the clinical activity of phase II-evaluable drugs on the basis of patterns of activity in the screen.

PROJECT DESCRIPTION

Major Findings:

(1) Development of neural networks (MecNet) capable of predicting mechanism of drug action on the basis of patterns of activity in the 60-cell line cancer drug screen. Six mechanisms of action were considered: alkylating agent, topoisomerase I inhibitor; topoisomerase II inhibitor, RNA/DNA antimetabolite, DNA antimetabolite, and anti-mitotic agent. The best network correctly predicted the category of 129 out of 141 drug vectors, a 65-fold improvement in the odds ratio over equal, random assignment to one of the categories or to "none of the above."

(2) Development of a program (DISCOVER) for identification of new structural and functional motifs among the tens of thousands of agents tested in the screen;

(3) Use of clustering in combination with neural networks and discriminant analysis to identify candidate cell lines for replacement in the screen (by breast, prostate, target-selected, target-transfected, and non-malignant lines, as appropriate).

(4) Preliminary predictions of the clinical activity of phase II-evaluable drugs on the basis of patterns of activity in the screen.

AIDS Research:

Formally, this is an application of information technology to the cancer drug development process, but analogous approaches to drugs for therapy of AIDS are planned.

SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS
NATIONAL CANCER INSTITUTE
1992

The Laboratory of Pathology is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. A fully accredited 4-year residency program in anatomic pathology is provided for 9 residents and 3 fellows. The Laboratory is divided into 10 sections:

Surgical Pathology Section (Dr. Maria J. Merino, Chief)
Pulmonary Pathology Section (Dr. William D. Travis, Chief)
Postmortem Pathology Section (Dr. Gitie S. Jaffe, Chief)
Cytopathology Section (Dr. Diane Solomon, Chief)
Ultrastructural Pathology Section (Dr. Maria Tsokos, Chief)
Biochemical Pathology Section (Dr. David D. Roberts, Chief)
Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
Gene Regulation Section (Dr. David L. Levens, Chief)
Office of the Chief (Dr. Lance A. Liotta, Chief)

All sections conduct investigative work and provide research opportunities for the residents. Investigative work completed or in progress is listed by section as follows.

Surgical Pathology Section

The Surgical Pathology Section provides expertise and diagnostic services in the field of Anatomic Pathology for the Institutes and Clinical Center patients, and collaborates with the research staff in those investigations which involve the use and study of human pathological material. Approximately 6,000 surgical specimens and biopsies (more than 60,000 slides which include routine and a variety of special stains) were accessioned last year. These include more than 2,000 fresh human tissues. A tissue procurement nurse works in close collaboration with the surgical pathology staff and helps in the distribution of tissues to scientists throughout the NIH.

The members of the section also participate in a variety of teaching and interdepartmental conferences (Medicine Branch, Surgery Branch, etc.) in which patient diagnosis and modalities of therapy are discussed, assisting in this way, to provide better patient care. Other objectives of the Surgical Pathology Section include carrying independent research by the members of the section and providing a residency program in anatomic pathology.

The section also provides consultant services to the community as well as to pathologists throughout the country.

Dr. Merino, in collaboration with other members of the Surgical Pathology staff, is investigating the role of different tumor markers as prognostic tools in the diagnosis of breast, ovarian and thyroid cancer, as well as soft tissue sarcomas. Dr. Merino is currently evaluating a number of antibodies used as proliferative markers (Ki 67), antibodies against enzymes known to be important in progression to tumor invasion and metastases (collagenases), 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, utilizing immunohistochemical techniques and antibodies against laminin and collagen IV, is studying the correlation between rupture of basement membranes in early invasive cancers, and patient outcome.

Pulmonary Pathology Section

Dr. William Travis is conducting a detailed review of interstitial fibrotic lung disease. Lung biopsies from patients with idiopathic pulmonary fibrosis have been reviewed and the data are currently being analyzed. Biopsies from 48 patients with pulmonary histiocytosis X are also being studied. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings. A new fellowship has been established for training in pulmonary pathology.

Postmortem Pathology Section

Dr. Gitie Jaffe is studying the clonality and molecular marker expression of parathyroid adenomas.

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to the study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH. Molecular techniques are also being applied to the study of lung cancer.

The neuropathology service is integrated with the Surgical Pathology Section, the Postmortem Pathology Section, and the Ultrastructural Pathology Section. Neuropathology diagnostic (patient care) service and teaching (of pathology residents) are provided. Approximately 250 neurosurgical specimens were

examined last year. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations, including dementia, pituitary adenomas, PML and gliomas.

Cytopathology Section

The Laboratory of Pathology provides complete services in anatomic pathology for the Clinical Center, a 550 bed research hospital. The Cytopathology Section provides diagnostic service on exfoliative cytology, fine needle aspiration cytology, and immunocytochemistry. The section accessions approximately 4,000 specimens per year. 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 training.

Cytopathology research efforts most recently have been directed towards the application of immunocytochemistry in diagnostic cytopathology. Lymphoid markers have been utilized to differentiate reactive processes from lymphoma, as well as to subtype lymphomas when possible. Several monoclonal antibodies have been evaluated for specificity and sensitivity for carcinoma cells versus reactive mesothelial cells in the diagnosis of metastatic carcinoma in cavity fluids. Currently under investigation is the use of *in situ* hybridization as an ancillary diagnostic technique for cytology.

Through a national consensus conference, this section has established the "Bethesda" system for standardizing the classification and reporting of PAP smears.

Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year approximately 200 cases were accessioned; over 150 were processed and diagnosed. This facility provides diagnostic training and clinical research opportunities for residents and fellows. Dr. Maria Tsokos has been doing diagnostic electron microscopy and at the same time has provided diagnostic light and electron microscopic consultation services for the Pediatric Oncology Branch (POB) at the NCI with which she has established a close working relationship. She also conducts an active research program in the characterization of small round cell tumors of childhood.

Small round cell tumors comprise one of the most difficult group of tumors in the differential diagnosis of cancer in children and young adults. Therapeutic modalities depend on recognition of distinct histopathologic entities and vary significantly with tumor type. Furthermore, modified therapeutic approaches have been adopted for tumors of similar histogenetic

origin that show different biologic aggressiveness. Therefore, there is increasing need for a new methodology, contributing to improvement of the diagnostic and predictive accuracy.

Dr. Tsokos has focused on: (a) the identification of markers and employment of techniques that help in the diagnosis and histogenetic characterization of Ewing's sarcoma, primitive neuroectodermal tumors (PNET), and rhabdomyosarcoma and (b) the definition of histologic, biologic and other factors to predict biologic aggressiveness.

Immunohistochemical studies with various neural and muscle markers evaluated by Dr. Tsokos have shown a great value of muscle and the limited value of neural markers in the differential diagnosis of neural from muscle tumors. Antibodies against histocompatibility class I antigens, such as those against $ß_2$-microglobulin have proven very useful in the discrimination of peripheral PNET (positive) from neuroblastomas (negative, except for ganglion cells, or stage IVs tumors).

A monoclonal antibody raised against surface antigenic determinants of Ewing's sarcoma (HBA.71) is being investigated by Dr. Tsokos in 100 cases of small round cell tumors of childhood with diverse histogenesis. The antibody has been provided by Dr. Link. Preliminary data support differential staining of Ewing's sarcoma/PNET (positive) from neuroblastoma (negative). The main goal of the study, however, is to discriminate between primitive rhabdomyosarcoma and osseous or mainly extraosseous Ewing's sarcoma. The study was initiated after review of treatment protocols existing in the Intergroup Rhabdomyosarcoma Study. Dr. Tsokos participated in this review as an invited consultant. Markers for separation of extraskeletal Ewing's sarcoma from primitive rhabdomyosarcoma are crucial for treatment of patients in the appropriate protocols.

Another antibody against MyoD1 protein, the product of one of the several identified muscle determination genes, has been used by Dr. Tsokos to stain frozen sections of round cell tumors of childhood. The antibody was found specific, although equally sensitive to desmin, in the diagnosis of rhabdomyosarcoma. The results of immunostaining paralleled those of MyoD1 gene expression detected by Northern blot analysis.

Recently, a technique by which loss of heterozygosity is identified in paraffin sections and another one by which translocations are detected in paraffin sections by fluorescence staining (FISH method) have been described. Both methods allow the study of archival material with clinicopathologic correlations and will be employed by Dr. Tsokos in the near future to answer relevant questions in childhood neoplasia.

P-glycoprotein (Pgp) has been associated with a multidrug-resistant (MDR) phenotype in a wide variety of animal and human tumor cell lines and clinical tumor specimens. Pediatric tumors, however, have not been included in the studies reported so far. Dr. Tsokos initiated and participated in several studies involving Pgp expression by immunohistochemistry using the C219, JSB1, and MRK-16 antibodies, as well as mdr-1 gene expression by Northern blot

analysis and *in situ* hybridization. Immunohistochemical staining of Ewing's sarcoma, PNET and rhabdomyosarcoma showed positive staining even before treatment, and no increased levels of expression after treatment, suggesting intrinsic Pgp expression, and lack of implication of the mdr gene in treatment failures occurring in this group of tumors. The mdr-1 gene was also expressed in several neuroblastomas, although lower levels than those observed in drug resistance. A subtle correlation of mdr-1 gene and Pgp expression with differentiaton was demonstrated in neuroblastomas, although not as evident as seen in neuroblastoma cell lines treated with retinoic acid. The study of the mdr-gene expression in neuroblastoma will soon appear in one of the issues of the American Journal of Pathology. Dr. Tsokos has also participated in collaborative studies related to elucidation of multidrug resistance mechanisms in tumors with investigators from the Medicine Branch at the NCI.

In addition, small round cell tumors of childhood have served as models to study mechanisms of human neoplasia in general, and have been used to verify the existence of the hypothesized tumor suppressor genes, which are not only involved in childhood, but also in adult neoplasia.

Dr. Tsokos has initiated studies of differentiation in rhabdomyosarcoma, designed to provide information with which to determine the relevance of phenotypic similarity of this tumor with developmental stages of normal skeletal muscle. The ultimate goal would be to create a model for studying pathogenetic mechanisms of this tumor development and identify factors with an inhibitory or differentiating effect, which may lead to therapeutic trials. It was found that human rhabdomyosarcoma retains the capacity to differentiate *in vitro*, similarly to normal skeletal muscle and that differentiation can be induced by 5-azacytidine (5-aza), an agent normally used in the treatment of malignancies. However, differentiation of rhabdomyosarcoma *in vitro* did not result in complete cell cycle withdrawal, although the differentiation effect of 5-aza appeared to be cell cycle-specific (exaggerated differentiation after the second dose, added 7 days after the first). Increased levels of $MycD_1$ RNA and protein expression after 5-aza treatment suggested activation of muscle determination genes as a possible mechanism of action.

Transforming growth factor (TGF-ß), evaluated in the same system, was found to inhibit morphologic differentiation, similar to its inhibitory effect in normal myogenesis, but without remarkable changes in the levels of $MyoD_1$ gene expression. Immunohistochemical studies with antibodies against TGF-ß1 and TGF-ß3 in round cell tumors of childhood were performed in collaboration with Dr. Sporn's laboratory and the levels of TGF-RNA expression were evaluated by Northern blot analysis of several cell lines. It was found that rhabdomyosarcoma expressed the highest TGF-ß mRNA and protein levels and neuroblastoma the lowest, with Ewing's sarcoma and PNET exhibiting intermediate and more variable levels of expression. In neuroblastoma, increased staining of differentiating ganglionic cells suggested a role for TGS-ß in the differentiation of this tumor and led to initiation of experiments in the section to evaluate levels of expression in response to treatment-induced differentiation *in vitro*. In rhabdomyosarcoma, a possible autocrine role of action has been speculated on the basis of increased synthesis and the observed inhibitory effects on myogenesis. The mechanism of

action of TGF-ß in rhabdomyosarcoma is currently evaluated in the section using blocking monoclonal antibodies and cDNA probes to detect levels of expression TGF-ß-receptor genes.

Biochemical Pathology Section

The Biochemical Pathology Section, under Dr. David Roberts, is conducting research on the function of complex carbohydrates in tumor cell adhesion and host-pathogen interactions and the role of the adhesive glycoprotein thrombospondin in tumor growth and metastasis. Current research projects in the section include: 1) identification and purification of tumor cell receptors for thrombospondin and characterization of the intracellular second messengers produced in response to thrombospondin binding to these receptors, 2) identification of peptide sequences in thrombospondin mediating tumor cell adhesion and migration, 3) characterization of thrombospondin and laminin interactions with sulfated glycolipids and proteoglycans and their role in regulation of angiogenesis and tumor growth and metastasis, and 4) structural analyses of novel complex carbohydrates expressed on human tumors or utilized as adhesion receptors by pathogenic micro-organisms.

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, whereas the amino-terminal domain mediates cell spreading and chemotaxis. The cell receptors recognizing these two regions of thrombospondin are under investigation. One class of receptors are sulfated glycoconjugates which bind to the amino-terminal domain of thrombospondin. A minor heparan sulfate proteoglycan that binds thrombospondin with high affinity was identified in two melanoma cell lines and purified by affinity chromatography on thrombospondin-Sepharose. An unusual sulfated glycolipid present only in melanoma cell lines that spread on thrombospondin was also found to bind thrombospondin. This glycolipid, purified from peripheral nerve, and a monoclonal antibody to the glycolipid specifically inhibit melanoma cell spreading on thrombospondin but not on fibronectin. To further define the mechanism of thrombospondin interactions with tumor cells, receptors for the carboxyl-terminus of thrombospondin are being characterized. Small cell lung carcinoma cells, which attach on thrombospondin but not on other adhesive proteins, will be used to identify specific thrombospondin receptors. Peptides from thrombospondin that inhibit adhesion of these cells and promote cell adhesion when coupled to a carrier protein are being used to characterize the regions of thrombospondin mediating cell adhesion and migration. The intracellular responses of cells to binding of thrombospondin to the two types of receptors are also being investigated. Reciprocal regulation of cyclic nucleotide and inositol phosphate levels has been found in melanoma cells exposed to thrombospondin. Experiments are in progress to determine the role of these changes in mediating the effects of thrombospondin on cell adhesion, growth, and motility.

Several approaches are being used to characterize the interactions of sulfated glycoconjugates with the adhesive proteins thrombospondin and laminin.

Suramin is a polysulfonated drug with several biological activities including inhibition of binding of some growth factors to cells, inhibition of tumor cell growth, and of glycosaminoglycan metabolism. Suramin also inhibits binding of thrombospondin and laminin to immobilized sulfatide, spreading of melanoma cells on thrombospondin and laminin, and cell attachment on laminin. However, suramin has no effect on cell attachment or spreading on fibronectin. Chemotaxis of melanoma cells to thrombospondin and laminin are also specifically inhibited by suramin. These results suggest a new mechanism for the observed antitumor activity of suramin based on its ability to inhibit interactions of tumor cells with laminin or thrombospondin in the extracellular matrix. A sulfatide-binding site on the globular end region of the long arm of laminin has been identified. This fragment is composed of two peptides that are covalently linked by at least one disulfide bond and encompass the carboxyl-terminal 394 amino acids of the A chain. The clusters of charged amino acid residues in the primary structure of these fragments are sufficient for heparin-binding activity but not sulfatide binding. The iodinated fragment bound specifically to melanoma and breast carcinoma cells. Both cell lines synthesize sulfated glycolipids that bind to laminin. In agreement with previous data that indicate a synergistic interaction of the sulfatide-binding domain with other laminin-binding sites on melanoma cells during attachment, the isolated sulfatide-binding fragment or sulfated polysaccharides that bind to this site significantly inhibited interaction of labeled intact laminin with melanoma and breast carcinoma cells in direct binding assays.

Recognition of host cell surface glycoconjugates or cell matrix proteins is a critical early step in initiation of infection by pathogenic microorganisms. Adhesive specificities of some Enterococcus species, *Candida albicans*, and elementary bodies of *Chlamydia trachomatis* are being examined. These were screened for binding to glycoproteins and glycolipids of known structure and to glycoconjugates isolated from target tissues to which the pathogens adhere and several novel adhesive specificities were identified. Where possible, inhibitors of each binding specificity will be identified using solid phase assays and then tested using *in vitro* cytoadherence assays and *in vivo* infection assays to determine the role of each in cytoadherence and initiation of infection.

Tumor Invasion and Metastases Section

Invasion and metastasis, the most life-threatening aspect of cancer is the culmination of a series of progression steps resulting in genetic changes over and above those required for uncontrolled proliferation. Expression of the metastatic phenotype depends on a balance between positive and negative regulatory gene products. Understanding the action of these gene products has led to new strategies for prognosis and therapy.

Dr. Mark Sobel is studying laminin receptors and their role in tumor cell metastasis. He has found a strong correlation between laminin receptor mRNA and protein levels and metastatic outcome for colon and breast cancer.

Dr. William Stetler-Stevenson is studying type IV collagenase, a metalloproteinase first identified by this section, which cleaves basement membrane type IV collagen at a specific locus, and is augmented in metastatic tumors. Negative regulation of type IV collagenase may be mediated through TIMP-2, a novel human metalloproteinase inhibitor recently identified by Dr. Stetler-Stevenson. The complete primary structure of TIMP-2 has been determined, and a full-length cDNA clone encoding TIMP-2 has been isolated. TIMP-2 binds to the latent form of type IV collagenase with a one-to-one molar stoichiometry and abolishes the catalytic activity of the activated enzyme. TIMP-2 may function as a tumor suppressor protein by inhibiting metalloproteinase activity required for invasion. TIMP-2 totally blocks the invasion of cancer cells through reconstituted basement membranes *in vitro*. *In vivo* TIMP-2 may arrest metastasis through inhibition of angiogenesis. Specific clinical applications of TIMP-2 could include the treatment of bone metastasis, because bone destruction is mediated by collagenases.

Progression to the metastatic phenotype may involve the loss of genes normally involved in development, morphogenesis, or differentiation. Dr. Pat Steeg, another investigator in the section, has obtained the full-length cDNA for NM23, a novel gene for which RNA levels are reduced in high metastatic potential murine melanoma cell lines and human tumor cells. These investigators have identified the 17 kDa protein product of this gene and find that the protein is virtually identical to the awd protein involved in Drosophila development and morphogenesis. The NM23 protein also shows a high degree of homology to a gene product involved in the differentiation of Dictyostelium. Mutation or allele loss associated with NM23 may lead to a disordered state favoring malignant progression. NM23 allele loss has been identified in a variety of human tumors.

Loss of NM23 expression in breast cancer is associated with a highly significant reduction in survival. Transfection of NM23 cDNA leading to augmented NM23 protein production abrogates metastasis by a non-immunologic mechanism in rodent melanoma models. Recent studies indicate that the NM23 protein is an NDP kinase. NDP kinases transfer an inorganic phosphate group from a donor molecule ATP to an acceptor molecule GDP producing GTP. The functional role of NM23 NDP kinase activity is under investigation. As a cancer marker, NM23 may provide a new approach to predicting the metastatic aggressiveness of an individual patient's tumor. Agents which modulate NM23 expression or function, or mimic its action, may have therapeutic potential.

Locomotion is a necessary component for tumor cell invasion. Members of the section have also been studying the transducer systems involved in the stimulated motility of invasive cancer cells. Dr. Mary Stracke is cloning the gene for a potent new motility stimulating cytokine, autotaxin. Autotaxin is a 120,000 dalton glycoprotein that has recently been purified and partially sequenced from the human melanoma cell line, A2058. This cytokine stimulates a pertussis toxin-sensitive motility response in these same cells at concentrations from 100 pM to 20 nM. Anti-peptide antibodies have been produced in rabbits against selected autotaxin peptides that recognize the protein on immunoblots. These affinity-purified antibodies are being utilized for biochemical and histochemical studies of autotaxin. Dr. Beckner has

cloned the gene for a new transmembrane protein which regulates tumor cell locomotion. Cytokine mediated stimulation of human melanoma cell motility was found by Dr. Aznavoorian and Dr. Savarese to operate through a pertussis toxin sensitive G protein pathway which regulates arachadonic acid and calcium fluxes. Dr. Aznavoorian has developed a new system to measure and isolate individual pseudopodia. This has led to new insights into the mechanism of pseudopodial protrusion and the role of G proteins, cytoskeleton and receptors in this process.

Screening compound which inhibits this specific pathway has led Dr. Kohn to identify a new signal transduction inhibitor which blocks tumor cell cytokine stimulated growth and motility. The inhibitor, termed CAI, is a substituted triazole which constitutes a new approach to cancer therapy. In animal models using a variety of human tumors, including melanoma, CAI has produced primary tumor and metastasis regression following oral administration. CAI has low toxicity, in studies to date, and is being considered as a potential chemopreventive agent. Clinical phase I trials for treatment of refractory cancers began in March, 1992.

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 1000 cases per year in consultation from the general 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 Surgery Branch, NCI.

Immunophenotypic analyses are performed using frozen section immunohisto-chemistry and flow cytometry. The flow cytometry laboratory utilizes a FACS scan and a FACS star, and is supervised by Dr. Maryalice Stetler-Stevenson. The section also offers studies in applied molecular diagnosis, using DNA and RNA probes. The diagnostic molecular biology laboratory is supervised by Dr. Mark Raffeld. These facilities are all integrated in the fellowship program in hematopathology.

The Hematopathology Section has published a number of important studies on the clinicopathologic and immunophenotypic aspects of malignant lymphoma. Dr. Jaffe described a unique association of nodular lymphocyte predominant Hodgkin's disease 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. None of the patients developed disseminated large cell lymphoma. Immunophenotypic and molecular genetic analysis was suggestive of a B-cell derivation for the proliferating cells, and further supports a B-cell origin for the L and H cell of lymphocyte predominant Hodgkin's disease. 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.

The section has continued its analyses of the angiocentric immunoproliferative lesions. Most recently, a molecular biologic analysis was completed utilizing T-cell receptor and immunoglobulin gene probes, as well as probes for the Epstein-Barr virus. This study demonstrated a surprising absence of T-cell gene rearrangement in most cases, but found a high incidence of EBV. In two cases the EBV appeared to be clonal, based on analysis of episomal terminal repeat regions.

In several papers, including clinical, pathologic, immunophenotypic, and molecular analysis, lymphocytic lymphoma of intermediate differentiation (IDL) or mantle zone lymphoma was confirmed as a distinct entity. A blastic variant of the disease associated with a more aggressive clinical course was described. The proliferative rate of the tumors as measured by Ki-67 positivity correlated with mitotic index and was associated with adverse survival when elevated. Drs. Raffeld and Medeiros demonstrated a high incidence of bcl-1 translocations in IDL, suggesting that this may be a reliable tumor marker.

Dr. Jaffe has continued to study the pathologic features of HTLV-I associated lymphomas. In selected populations where HTLV-I is endemic, such as 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. Current studies conducted in collaboration with Dr. Mark Raffeld explore the role of the polymerase chain reaction (PCR) technique to detect HTLV-I viral sequences. These data are correlated with serologic studies for HTLV-I associated antibodies.

Dr. Maryalice Stetler-Stevenson has demonstrated that frequent relapse of follicular lymphoma, the major obstacle to cure, is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. To detect occult lymphoma, she has specifically amplified the joined bcl-2/JH DNA sequences created by the t(14;18) translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (polymerase chain reaction, PCR), she can detect 1 copy of bcl-2/JH, which is four orders of magnitude more sensitive than flow cytometry or Southern blot restriction analysis. Genomic DNA sequence analysis of four lymphomas confirmed that the size of the

amplified fragment serves as a unique tumor marker. Direct application to clinical samples has demonstrated lymphoma cells which were otherwise undetectable.

Dr. Raffeld has completed a molecular analysis of small non-cleaved cell lymphomas, further subclassified as sporatic Burkitt's type and non-Burkitt's. These studies confirm a molecular basis for the morphologic subclassification of small non-cleaved cell lymphoma. Whereas 17 of 18 cases of Burkitt's lymphoma showed a c-myc rearrangement, no case of non-Burkitt's lymphoma contained such a molecular abnormality. In 3 cases, a bcl-2 rearrangement suggesting the presence of a 14;18 translocation was identified, indicating a relationship of the non-Burkitt's subtype follicular center cell neoplasms. In the Burkitt's lymphomas, the molecular breakpoint regions were further mapped using specific probes to the immunoglobulin heavy chain gene regions: JH, switch α, switch μ, or cμ. Three cases which demonstrated a rearrangement involving the switch a region were associated with a particularly poor prognosis.

Dr. Raffeld, in collaboration with Dr. Han Van Krieken, a Guest Worker in the Hematopathology Section, completed a study of the molecular genetics of gastrointestinal non-Hodgkin's lymphomas. This study found a low incidence of bcl-1 and bcl-2 translocations, arguing for a different pathogenesis for gastrointestinal non-Hodgkin's lymphoma from that of node-based non-Hodgkin's lymphoma. A rearrangement of the c-myc gene was found in 6 of 8 Burkitt-like lymphomas of the intestine. In 5 of these 6 cases, a chromosomal translocation t(8;14) with an unusual breakpoint was demonstrated by co-migration of the rearranged c-myc and a rearranged JH sequence. C-myc rearrangements were also found in 6 of 12 large cell or high grade mucosa-associated lymphomas of the stomach. However, no co-migration of c-myc and immunoglobulin heavy chain gene sequences were found. The patterns of c-myc rearrangements in gastric large cell lymphoma and ileocecal Burkitt's lymphoma are noteworthy and suggest a different and distinct pathogenesis for these two aggressive lymphomas.

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.

Application of an exonuclease assay developed in this laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA to the c-myc gene has revealed multiple cis- and trans-elements both upstream and downstream of the major c-myc promoters P1 and P2. Four elements have been studied extensively. First, because cessation of c-myc transcriptional initiation has been shown to occur during pharmacologically induced differentiation of monomyelocytic leukemia cell lines and because this event

appears to be a prerequisite for differentiation, experiments to identify a differentiation inducible repressor or a differentiation repressible activator were performed. Modulation of a factor as detected by loss of binding activity to a site 1500 bp upstream of promoter P1 was noted. The precise binding site was defined by deletional and mutational analysis. Functional transfection studies have indicated that this binding site serves as a positive element in undifferentiated leukemia cells. Following differentiation, the far upstream element, designated FUSE, ceases to stimulate c-myc expression. A 75 kD protein binding to the FUSE has been purified and subjected to micro-sequence analysis allowing the cloning of a candidate for a gene encoding the FUSE binding factor. The characterization of the FUSE binding protein, its gene, and its biological regulation are in progress.

Another region upstream of c-myc serves as a negative cis-element. We have demonstrated that the sequence responsible for this negative control binds two factors with completely overlapping binding sites. One of these factors, AP1, is known to contain, in part, a complex of two proto-oncogene products, the c-jun and c-fos proteins. The other factor is an octamer binding protein. The relative contributions of AP1 and octamer binding proteins to the activity of this element are under investigation. To follow up on the role of AP1 as a potential modulator of c-myc expression during differentiation, we have commenced a search for new members of the AP1 family whch may potentially bind to the c-myc negative element.

Previously, in collaboration with Dr. Maria Zajac-Kaye of the Medicine Branch, we have located a cis-element in intron 1 of the human c-myc gene and demonstrated that this element binds a nuclear protein and that the element is mutated in most Burkitt's lymphomas. We have extended these investigations by the identification of a 140 kD phosphoprotein responsible for this binding activity. Importantly, phosphorylation appears to be necessary for strong binding to the myc intron sequence. The analysis of the regulation of phosphorylation of this factor as well as functional analysis of the cis-element are underway. Additionally, a second protein component of the specific DNA-binding complex has been identified as a protein of approximately 35 kD. The role of this second factor in c-myc regulation is under investigation.

One hundred bases upstream of the c-myc promoter is an element composed of multiple repeats of the sequence CCCTCCCCA. We have shown this region to contain a positive acting, orientation dependent cis-element capable of stimulating expression from heterologous promoters approximately 5-fold both *in vivo* and *in vitro*. Multiple proteins with novel properties bind to this element. The identificaton and characterization of these proteins promises to reveal new features governing c-myc expression.

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. The gibbon T-cell lymphoma cell line MLA 144 strongly

transactivates the GALV-Seato enhancer. Although this element contains an AP1 site, the factors which bind this site in T cells is distinct from known members of the fos/jun family. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not itself possess full specificity. The second component does not itself bind DNA, but upon forming a complex with the first component, confers greatly enhanced power to discriminate between different sequences. The regulation of the interaction of these elements appears to be an early event in T-cell activation. The cloning and characterization of these proteins are in progress.

Office of the Chief

Dr. Susan Mackem is interested in elucidating the mechanisms at the molecular level by which pattern formation is regulated during embryonic development. Using limb morphogenesis as a model system for pattern formation that is readily amenable to various experimental manipulations, Dr. Mackem is employing subtractive hybridization approaches to isolate cDNA clones for genes that are induced during pattern formation in the embryonic limb and that play potential roles in determining limb-type identity. Currently, subtracted libraries enriched for wing- and for leg-specific sequences have been generated and screening with wing- and leg-enriched cDNA probes is underway to isolate genes regulating differences in wing/leg pattern formation. As a second, more directed approach, the role of known gene families thought to have regulatory functions in development is being investigated in the context of limb morphogenesis. Using a PCR-based approach, a number of homeobox genes that are expressed in developing limb buds have been identified. Two of these genes are novel non-Antennapedia homeobox genes with homeodomain sequences of some similarity to *Drosophila Abd-B (Ghox 4.7)* and *D11* (L5) respectively, and their spatiotemporal expression domains during development have been analyzed using *in situ* hybridization techniques on both sectioned embryos and on whole mount embryos.

Ghox 4.7 is expressed in a highly posteriorly restricted domain of the early limb bud correlating with the position of a functional zone regulating anterior-posterior (A-P) pattern and hence suggesting a role for this gene in patterning along the A-P axis. Whole mount *in situ* hybridization with several of the chick *Hox 4* cluster genes shows that the very posteriorly restricted *Hox 4* genes also display quantitative and qualitative expression differences between wing and leg buds in the chick embryo, unlike the mouse embryo. These differences may be related to modification of the avian wing from the general tetrapod limb pattern for flight and so the limb-type differences in chick *Hox 4* expression may also reflect a role in regulating A-P pattern.

The second gene (L5) has a highly restricted expression domain along the proximo-distal (P-D) axis, 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 tip. At later stages (st 26-28), the expression is localized more proximally, and is restricted to the region of

the anterior distal zeugopod (radius or tibia) and proximal autopod (carpals or tarsals). This type of expression is consistent with the known progressive determination of structures along the P-D axis in a proximal to distal sequence and suggests a role for L5 in the determination of positional identity, and hence pattern, (for example of the wrist/ankle) along the P-D axis. Microsurgical manipulations (apical ridge excisions/grafts; retinoid treatment) are currently underway to analyze the expression pattern of this gene when the developmental program (pattern) is experimentally altered. L5 is also expressed in two other locations in the very early embryo; the anlage of the pineal gland, and in the portion of the notochord adjacent to paraxial mesoderm that has not yet become segmented into somites (which recedes caudally as development proceeds).

Long-term experiments to determine the function of these genes are currently underway, and include characterizing the effects of ectopic overexpression as well as ablation of expression of these genes using transgenic technology in mice, and avian retroviral expression vectors for transient expression experiments in chick embryos. Biochemical approaches are also being employed to identify the downstream "target" genes that are regulated by *Ghox 4.7* and L5 during limb morphogenesis.

Dr. Kathleen Kelly is investigating the consequences of mitogen-mediated signals to T cells. She has isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. Primary sequence analyses have been completed on several clones, and selected clones are being studied in more detail. Several interesting functional classes of growth-regulated proteins have been revealed including a structurally unique class of tyrosine phosphatase, a novel GTP-binding protein associated with the endoplasmic reticulum, and a cell surface receptor with seven transmembrane regions that couple signal transduction through G proteins. These proteins are being studied with regard to biochemical properties and potential physiological function.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00853-39 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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 MAN-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:

G. Jaffe	Expert	LP NCI
J. Taubenberger	Expert	LP NCI
D. Kleiner	Medical Staff Fellow	LP NCI
+E. Dean-Clower	Medical Staff Fellow	LP NCI
+H. Hollingsworth	Medical Staff Fellow	LP NCI
+K. Gardner	Medical Staff Fellow	LP NCI
+R. Doms	Medical Staff Fellow	LP NCI
+C. Moskaluk	Medical Staff Fellow	LP NCI
+T. Giordano	Medical Staff Fellow	LP NCI
+S. Barksdale	Medical Staff Fellow	LP NCI
+M. Roth	Medical Staff Fellow	LP NCI
+M. Buck	Medical Staff Fellow	LP NCI
*J. Stern	Consultant in Dermatopathology	LP NCI
D. Katz	Consultant in Neuropathology	LP NCI

Objectives:

(a) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH;

(b) to carry out independent research;

(c) to provide a residency program in anatomic pathology; and

(d) to collaborate with investigators in research involving the use and study of human materials

The proposed course of research includes (a) continuing to provide the services described; (b) increasing the interaction with the clinical branches in the design and evaluation of protocols; (c) improving the opportunities for the resident staff to participate in teaching, conferences and seminars, and providing elective periods to be spent accomplishing research projects with the senior staff; and (d) implementing data retrieval programs.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

Histologic and immunohistology studies will be performed as part of the following clinical protocols: 1) Dose intensive chemotherapy in locally advanced and metastatic breast cancer; 2) Use of monoclonal antibody CC49 to treat breast cancer; 3) Combination radioiodine and adriamycin for follicular thryoid cancer; 4) Treatment of stage I and II carcinoma of breast, mastectomy vs. lumpectomy; and 5) Phase II evaluation of suramim in advanced stage carcinoma of prostate.

+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:

Merino MJ. Special variants of papillary thyroid carcinoma. Ann Intern Med 1991;115:133-47.

Pesce C, Merino MJ, Chambers J, Nogales, F. Endometrial carcinoma with trophoblastic differentiation: An aggressive form of uterine cancer. Cancer 1991;68:1799-1802.

Merino MJ. Vaginal cancer: The role of infectious and environmental factors. Am J Obstet Gynecol 1991;165:1255-62.

Axiotis CA, Guarch R, Merino MJ, Laporte N, Neumann RD. P-glycoprotein expression is increased in human secretory and gestational endometrium. Lab Invest 1991;65:577-81.

Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM. Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. N Engl J Med 1991;15:33-42.

Kragel P, Devaney K, Merino MJ. Struma ovarii with peritoneal implants. A case report with lectin histochemistry suggesting origin from ovarian serosal epithelium. Surg Pathol 1991;4:274-81.

Barth RJ, Danforth DN, Jr, Venzon DJ, Straus KL, d'Angelo T, Merino MJ, Gerber L. Level of axillary involvement by lymph node metastases from breast cancer is not an independent predictor of survival. Arch Surg 1991;126:574-7.

Merino MJ, Monteagudo C, Neumann RD. Monoclonal antibodies for radio-immunoscientigraphy of breast cancer. Nucl Med Biol 1991;18:437-43.

Kenney RT, Kwon-Chung KJ, Waytes AT, Melnick DA, Pass HI, Merino MJ, Gallin JI. Successful treatment of systemic *Exophiala dermatitidis* infection in a patient with chronic granulomatous disease. Clin Infect Dis 1992;14:235-42.

Spencer WF, Linehan WM, Walther MM, Haas GP, Lotze MT, Topalian SL, Yang JC, Merino MJ, Lange JR, Pockaj BA, Rosenberg SA. Immunotherapy with interleukin-2 and α-interferon in patients with metastatic renal cell cancer with *in situ* primary cancers: A pilot study. J Urol 1992;147:24-30.

Hobbs S, Neumann RD, Merino MJ, Gunzenhauser J, Carrasquillo JA. Localization of Tc-99m MDP in cystodsarcoma phyllodes. Clin Nucl Med 1992;17:58-60.

Anglard P, Trahan E, Liu S, Latif F, Merino MJ, Lerman MI, Zbar B, Linehan WM. Molecular and cellular characterization of human renal cell carcinoma cell lines. Cancer Res 1992;52:348-56.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09145-08 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
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 MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece 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 wide range of clinicopathologic investigations.

Major Findings:

The neuropathology service continues to function: (1) to provide a specialized diagnostic service for neurosurgical and autopsy material from NIH patients; (2) to use this material to carry out clinicopathologic studies of primary neurologic disease and neurologic complications of systemic disease, and to (3) teach resident trainees in anatomic pathology the fundamentals of neuropathology; (4) to assist, in collaborative fashion, basic investigators who desire to study human nervous tissue.

Autopsy: As in previous years, the brain was examined in approximately 75% of all autopsies, and approximately one-half of these manifested significant primary or secondary neurologic findings. Current care material includes dementia and other degenerative neurological diseases, AIDS (predominantly pediatric), 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 250 neurosurgical specimens are examined yearly, including both submitted and in-house cases. Approximately 25 intra-operative frozen section consultations are provided yearly. Current case material includes pituitary adenomas, metastatic tumors, electrocorticographically-guided resections for temporal lobe seizures, and spinal hemangioblastomas, as well as 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: study of adenomas, particularly in Cushing's disease (NICHD, NINDS).

3. AIDS (pediatric): diagnosis of AIDS encephalopathy.

4. Multiple sclerosis: correlation of acute lesions with neuroimaging; study of non-MS lesions mimicking MS on MRI (case reports).

5. Neuroimaging: *in vitro* NMR characteristics of normal brain tissue at autopsy.

6. HTLV-1-associated myelopathy: clinicopathologic study, correlation with PCR.

Publications:

Crittenden, MD, Roberts CS, Rosa L, Vatsia SK, Katz D, Clark RE, Swain JA. Brain protection during circulatory arrest. Ann Thorac Surg 1991;51:942-7.

Oldfield E, Doppman JL, Nieman LJ, Chrousos GP, Miller DL, Katz DA, Cutler GB, Jr., Loriaux DL. Bilateral inferior pestrosal sinus sampling with and without corticotropin releasing hormone for the differential diagnosis of Cushing's syndrome. N Engl J Med 1991;325:897-905.

Poirier MC, Reed E, Litterst CL, Katz D, Gupta-Burt S. Persistence of platinum-ammine-DNA adducts in gonads and kidneys of rats and multiple tissues from cancer patients. Cancer Res 1992;52:149-53.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09192-03 LP

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Histologic Changes in Renal Cell Carcinoma After LAK Therapy

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:	S. Rosenberg	Chief, Surgery Branch	SB NCI
	M. Linehan	Chief, Urology Section	SB NCI
	T. Giordano	Medical Staff Fellow	LP NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Pathology

SECTION: Surgical Pathology Section

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

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

The resected specimens of patients with renal cell carcinoma that have received immunotherapy will be evaluated histologically and immunohistochemically and compared with the renal cell cancers of patients which did not receive the same modality of treatment.

GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09193-03 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Malignant Changes Associated with Sclerosing Adhesions

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

PI: M. Merino Chief, Surgical Pathology Section LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-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.)*

Sclerosing adenosis is a proliferative breast lesion with pseudoinvasive features frequently misdiagnosed as infiltrating carcinoma. The purpose of this study will be: 1) evaluate the premalignant potential of this lesion; 2) its association with carcinoma and 3) evaluate the integrity of the basement membrane. Patients with sclerosing adenosis in which *in situ* cancers develop are probably at a much higher risk to evolve to an invasive cancer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09359-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Prognostic Markers in Soft Tissue Sarcomas

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:	S. Rosenberg	Chief, Surgery Branch	SB NCI
	J. Yang	Medical Officer	SB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Low-grade sarcomas are known to behave in an indolent fashion, with potential for late local recurrences. Proliferative markers will be used in an attempt to recognize these low-grade sarcomas that behave in an aggressive fashion and not only recur early but produce distant metastases. A clinicopathologic correlation will be done.

PHS 6040 (Rev. 1/84)

GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09361-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

P-Glycoprotein Expression in Breast Cancer

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

PI:	M. Merino	Chief, Surgical Pathology Section	LP NCI
OTHER:	V. Monterroso	Visiting Fellow	LP NCI
	K. Cowan	Chief, Medical Breast Cancer Section	MB NCI
	J. O'Shaughnessy	Senior Staff Fellow	COP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-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.)*

The multidrug-resistance (MDR) gene product, P-glycoprotein (P170) has been known to be increased in tumors resistant to chemotherapeutic drugs. We will evaluate the presence of P-glycoprotein in cases of breast cancer, utilizing immunohistochemical techniques. Biopsies obtained before and after treatment will be evaluated, utilizing commercial antibodies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09362-01 LP

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Phase I Study of IV ^{177}LU Murine CC49 Patients with Breast Cancer

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

PI:	J. O'Shaughnessy	Senior Staff Fellow	COP NCI
OTHER:	K. Cowan	Chief, Medical Breast Cancer Section	MB NCI
	J. Schlom	Chief, Immunology Section	LTIB NCI
	M. Merino	Chief, Surgical Pathology Section	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Pathology

SECTION: Surgical Pathology Section

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

TOTAL MAN-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.)*

The purposes of this study are:

1) to determine the presence of the monoclonal antibody CC49 in tissues;

2) to study the toxicity of ^{177}Lu CC49 in patients with advanced adenocarcinoma

3) to study the ability of CC49 to image known metastases

PHS 6040 (Rev. 1/84)

GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09166-05 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Pathology of Interstitial Pulmonary Fibrosis

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

PI:	W. Travis	Chief, Pulmonary Pathology Section	LP NCI
OTHER:	V. Ferrans	Chief, Ultrastructure Section	IR PA NHLBI
	R. Crystal	Chief, Pulmonary Branch	IR PB NHLBI

COOPERATING UNITS *(if any)*

Pulmonary Branch, NHLBI

LAB/BRANCH

Laboratory of Pathology

SECTION

Pulmonary Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	X

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

A detailed pathologic review of the NIH (Pulmonary Branch, NHLBI) experience with pulmonary interstitial fibrosis is being performed to investigate potential new approaches to the diagnosis and pathologic subclassification of interstitial fibrosis. Recent reports have described newly recognized forms of interstitial fibrotic lung disease previously classified as idiopathic pulmonary fibrosis suggesting a need for rethinking of traditional concepts of the pathology of pulmonary interstitial fibrosis.

The broad experience of the Pulmonary Branch, NHLBI, provides a rich resource of clinical and pathologic material which may provide the basis for recognition of new prognostically significant forms of interstitial lung fibrosis.

Lung biopsies from 80 patients with idiopathic pulmonary fibrosis have already been reviewed and the data are currently being analyzed. Biopsies from 48 patients with pulmonary histiocytosis X are also being written up. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.

Lung specimens from 26 patients with Churg-Strauss syndrome have been collected and are being analyzed for publication.

Publications:

Travis WD, Kwon-Chung KJ, Kleiner D, Geber A, Lawson W, Pass HI, Henderson D. Unusual aspects of allergic bronchopulmonary fungal disease: Report of two cases due to *Curvularia* infection associated with allergic fungal sinusitis. Hum Pathol 1991;22:1240-8.

Travis WD, Linnoila RI, Hitchock CL, Cutler GB, Chrousos G, Nieman L, Pass HI, Doppman JL. Neuroendocrine tumors of the lung with proposed criteria for large cell neuroendocrine carcinoma: an ultrastructural, immunohistochemical, and flow cytometric study of 35 cases. Am J Surg Pathol 1991;15:529-53.

Hoffman GS, Kerr GS, Leavitt RY, Hallahan CW, Lebovics RS, Travis WD, Rottem M, Fauci AS. Wegener's granulomatosis: a prospective analysis of 158 patients. Ann Intern Med 1992;116:488-98.

Rom WN, Travis WD. Lymphocyte macrophage alveolitis in nonsmoking individuals occupationally exposed to asbestos. Chest 1992;101:779-86.

Feuerstein IM, Jicha DL, Pass HI, Chow CK, Chang R, Ling A, Hill SC, Dwyer AJ, Travis WD, Horowitz ME, Steinberg SM, Frank JA, Doppman JL. Pulmonary metastases: MR imaging with surgical correlation--a prospective study. Radiology 1992;182:123-9.

Jaffe ES, Travis WD. Lymphomatoid granulomatosis and lymphoproliferative disorders. In: DeRemee R, Lynch JP, eds. Immunologically mediated pulmonary disease. Philadelphia: JB Lippincott Co, 1991;274-301.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09365-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Pulmonary Pathology

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

PI: W. Travis Chief, Pulmonary Pathology Section LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Pulmonary Pathology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The section of Pulmonary Pathology, together with the Cytopathology Section, Hematopathology Section, Postmortem Section, Surgical Pathology Section, and Ultrastructural Pathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material.

The pulmonary section provides diagnostic services for several pulmonary groups at the NIH including the NIAID (Dr. Fauci) for vasculitis, the NHLBI, Pulmonary Branch (Dr. Crystal) for interstitial lung disease, Critical Care (Dr. Masur) for lung disease in immunocompromised patients; and the NCI, Surgery Branch (Dr. Pass) for lung cancer and malignant mesothelioma protocols. The pathology for all interstitial lung disease patients with lung biopsies is presented at Dr. Crystal's weekly, Friday noon conference. Surgical and autopsy lung specimens are reviewed on a daily basis for these various pulmonary groups.

The pulmonary material is being utilized for research purposes to study lung cancer, malignant mesothelioma, and nonneoplastic interstitial lung disease. Electron microscopic and immunohistochemical techniques are being utilized to study these diseases. A prospective collaborative project has been undertaken in conjunction with Dr. Lance A. Liotta, Dr. Curtis C. Harris, and the lung cancer group at Mayo Clinic to investigate the molecular biology of lung cancer.

In addition, a collaborative working relationship has been set up with the Pulmonary and Mediastinal Branch at the Armed Forces Institute of Pathology. This has allowed study of a wide variety of rare and unusual lung diseases including neuroendocrine tumors of the lung, pulmonary epithelioid hemangioendotheliomas, sclerosing hemangiomas, primitive neuroectodermal tumors presenting in the lung, and sarcomatoid carcinomas of the lung.

Objectives:

The objectives of the Pulmonary Section are: (a) to provide diagnostic services in pulmonary pathology for the clinical research protocols conducted at NIH. This includes generating pathology reports for current cases and presenting the pathologic findings of lung biopsies at clinical conferences with clinicians and radiologists; (b) to carry out independent research; (c) to provide teaching to the NIH residency program in anatomic pathology, especially pulmonary pathology; (d) to collaborate with investigators in research involving the use and study of human materials; (e) to establish an internationally recognized pulmonary pathology training program for pathology residents in collaboration with the Armed Forces Institute of Pathology.

The proposed research program includes (a) continuing to provide the services described; (b) increasing interaction with clinical branches in the design and evaluation of protocols; (c) providing opportunities for residents to participate in teaching; and in research projects; (d) developing data retrieval systems for pulmonary pathology material.

Two major projects are currently in progress on the study of lung cancer:

1) A prospective collaborative project in conjunction with Dr. Lance A. Liotta, Dr. Curtis C. Harris, and the lung cancer group at Mayo Clinic to in vestigate the molecular biology of lung cancer. So far, tissue samples of lung tumor and nontumorous lung, as well as blood samples have been collected on 100 lung cancer patients. Each of these patients has completed a comprehensive epidemiologic questionnaire and has detailed information available regarding clinical history. Followup will become available through the Mayo Clinic department of Thoracid Disease. Molecular studies are underway in Dr. Harris' and Dr. Liotta's laboratories on these samples.

2) A retrospective study of 100 patients with neuroendocrine tumors from the AFIP files has been undertaken. The histologic features of these cases has been characterized and followup is being completed. This will provide important information about the clinical behavior of large cell neuroendocrine carcinoma, a tumor recently described by us at NCI. A prospective collaborative project has been undertaken in conjunction with Dr. Lance A. Liotta, Dr. Curtic C. Harris, and the lung cancer group at Mayo Clinic to investigate the molecular biology of lung cancer.

Publications:

Jensen JC, Choyke PL, Rosenfeld M, Pass HI, Keiser H, White B, Travis WD, Linehan WM. A report of familial carotid body tumors and multiple extra-adrenal pheochromocytomas. J Urol 1991;145:1040-2.

Mulé JJ, Jicha DL, Aebersold PM, Travis WD, Rosenberg SA. Disseminated human malignant melanoma in congenitally immune-deficient (bg/nu/xid) mice. J Natl Cancer Inst 1991;83:350-5.

Doppman JL, Nieman LK, Travis WD, Cutler GB, Norton JA, Chrousos GP, Loriaux DL. Massive macronodular adrenocortical hyperplasia: a rare cause of autonomous primary adrenal hypercortisolism. J Comput Assist Tomog 1991;15:773-9.

Fujita S, Puri RK, Yu Z-X, Travis WD, Ferrans VJ. An ultrastructural study of *in vivo* interactions between lymphocytes and endothelial cells in the pathogenesis of the vascular leak syndrome induced by interleukin-2. Cancer 1991;68:2169-74.

McKenzie R, Travis WD, Dolan S, Pittaluga S, Feuerstein IM, Shelhamer J, Yarchoan R, Masur H. The causes of death in patients with HIV infection: a clinical and pathologic study with emphasis on the role of pulmonary diseases. Medicine 1991;70:326-43.

Zeiger MA, Nieman LK, Cutler GB, Chrousos GP, Doppman JL, Travis WD, Norton JA. Primary bilateral adrenocortical causes of Cushing's syndrome. Surgery 1991;110:1106-15.

Lawrence JB, Friedman BS, Travis WD, Chinchilli VM, Metcalfe DD, Gralnick HR. Hematologic manifestations of systemic mast cell disease: a prospective study of laboratory and morphologic features and their relation to prognosis. Am J Med 1991;91:612-24.

Moran C, Travis WD, Rosado-deChristenson M, Koss MN, Rosai J. Thymomas presenting as pleural tumors. Am J Surg Pathol 1992;16:138-44.

Gaffey MJ, Traweek ST, Mills SE, Travis WD, Lack EE, Medeiros LJ, Weiss LM. Cytokeratin expression in adrenocortical neoplasia: an immunohistochemical and biochemical study with implications for the differential diagnosis of adrenocortical, hepatocellular, and renal cell carcinoma. Hum Pathol 1992;23:144-53.

Lack EE, Mulvihill JJ, Travis WD, Kosekewich HPW. Pediatric adrenal cortical neoplasms. Path Ann 1992; 27:1-53.

Metcalfe DD, Jensen RT, Siraganian RP, Travis WD. Mastocytosis. Update No 12. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, eds. Allergy: principles and practice, 3rd edition. 1991;1-13.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09364-01 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

PI: G. Jaffe Chief, Postmortem Pathology Section LP NCI

OTHER: D. Kleiner, C. Baker, P. Howley, D. Levens, L. Liotta, S. Mackem, T. O'Leary, A. Larner, A. Ginsberg, J. Taubenberger, D. Roth, H. Hollingsworth, K. Gardner, R. Doms, S. Barksdale, C. Moskaluk, T. Giordano, L. Abruzzo, L. Ritchie, A. Dock, J. Rainey, W. Roberts, D. Katz

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Postmortem Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
24	21	3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The Postmortem Pathology Section, along with the Cytopathology Section, Hematopathology Section, Surgical Pathology Section, and Ultrastructural Pathology Section, provide a complete service in Anatomic Pathology for the Clinical Center as well as other institute patients. In addition, when the use and study of human pathological material is requested by research staff of any of the institutes, the Postmortem Section makes every effort to collaborate with and/or supply the researchers with the human tissues upon approved request.

The autopsy material is utilized by staff and residents for research projects involving clinicopathological correlation and characterization of disease processes. Currently, several projects are on-going: immunohistochemical staining of various tissues with protease and nonmuscle myosin antibodies; wide organ sampling from all AIDS cases with PCR, immunohistochemical and histological analysis of HIV; clinicopathologic correlation in dementias; neuroimaging correlation in demyelinating disease; histologic correlation of *in vitro* NMR characterization of normal brain; and *in situ* hybridization for HSV in a patient with zoster myelitis/encephalopathy.

Additionally, a database of major autopsy diagnoses from 1982 through 1990 is being compiled, with pertinent historical information included.

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

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 00852-39 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
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:	Y. Hijazi	Expert	LP NCI
	D. Gagneten	Fellow	LP NCI
	C. Copeland	Cytotechnologist	LP NCI
	L. Galito	Biologist	LP NCI
	A. Wilder	Cytotechnologist	LP NCI
	E. Sanders	Bio. Lab. Technologist	LP NCI
	A. Macurdy	Chemist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Cytopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	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, immunocytochemistry, and flow cytometry.

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.

In conjunction with Dr. M.A. Stetler-Stevenson in the Hematopathology Section, we are initiating flow cytometry as an ancillary diagnostic technique in bladder washings. An example of one collaborative clinical research project involves clinical trials currently being conducted to study the use of the monoclonal antibody RFB4-RICIN A chain conjugate for refractory CD22 positive B-cell lymphoma. Our collaborative effort in this project involves the cytomorphologic evaluation of cytopathology specimens in order to: 1) document the presence of lymphoma and CD22 positivity prior to initiation of immunotoxin therapy and 2) monitor response by evaluating CD22 levels and presence of immunotoxin.

Another collaborative project with the Whitman-Walker AIDS clinic is evaluating the prevalence of cervical premalignant lesions in HIV infected women. A few reports in the literature have cited a high rate of dysplasias in HIV infected women. These findings, if substantiated, have implications for cervical screening recommendations for this population.

GPO 914-918

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 almost 20% per year. This modality has been embraced by clinicians as a minimally invasive technique which provides diagnoses rapidly and cost effectively, with minimal discomfort to the patient often obviating more invasive biopsy procedures.

Cases submitted by outside pathologists for consultation by the Cytopathology Section have increased in number by approximately 100% over previous years.

Cytological techniques are utilized in collaborative work with other sections and branches of NIH. For example, single cell tumor suspensions, tumor cell lines, and stimulated lymphocyte cultures are evaluated microscopically and by immunocytochemical techniques.

Publications:

Elwood L, King C, Colandrea J. Urinary cytology. In: Atkinson BF, ed. Atlas of diagnostic cytopathology. WB Saunders, 1992.

Elwood L, Dobrzanski D, Feuerstein I, Solomon D. *Pneumocystis carinii* in pleural fluid: The cytologic appearance. Acta Cytol 1991;35:761-764.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00897-09 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Immunocytochemistry as an Adjunct to Cytopathological Diagnosis

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	E. Jaffe	Chief, Hematopathology Section	LP NCI
	A. Macurdy	Chemist	LP NCI
	D. Gagneten	Biotechnology Fellow	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	.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.)*

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.

GPO 914-918

Major Findings:

Immunocytochemistry in the evaluation of lymphoid cell populations:

We have investigated 530 specimens, including 250 pleural and peritoneal effusions, 110 cerebrospinal fluids, and 170 fine needle aspiration specimens. We have found 254 cases to be positive for lymphoma and 160 to be reactive in nature. Of the 250 positive cases, 208 were diagnosed as monoclonal B cell proliferations on the basis of either κ or λ light chain but not both. A diagnosis of T cell lymphoma was made in 41 cases on the basis of aberrant marker phenotype or TdT positivity. Acute nonlymphocytic leukemia was diagnosed in three cases and Hodgkin's disease in two cases.

The application of immunocytochemistry to cytology specimens is an extremely valuable adjunct in the diagnosis of hematopoietic malignancies. Definitive cytological diagnosis of relapse/recurrence of disease guides clinical treatment of these patients. Particularly in the setting of HIV-associated lymphoma, unusual sites of initial presentation and/or the debilitated condition of many patients may preclude more invasive tissue biopsy diagnostic techniques. In these cases, a definitive cytopathologic diagnosis obviates the need for more invasive diagnostic procedures.

Publications:

Davis JL, Solomon D, Nussenblatt RB, Palestine AG, Chan CC. Immunocytochemical staining of vitreous cells: Indications, techniques, and results. Ophthalmology 1992;99:250-56.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09153-06 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Cytophenotypic Analysis of Tumor Suspensions and TIL Cultures in Immunotherapy

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	S. Topalian	Surgery Branch	SB NCI
	S. Rosenberg	Chief, Surgery Branch	SB NCI
	J. Yannelli	Surgery Branch	SB NCI
	A. Macurdy	Chemist	LP NCI

COOPERATING UNITS *(if any)*

Surgery Branch, DCT

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	.15	.15

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

D

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

Clinical trials employing the adoptive transfer of expanded tumor infiltrating lymphocytes to patients with metastatic disease are currently underway under the direction of the Surgery Branch, NCI. Our collaborative effort in this project involves immunocytochemical analysis of tumor cell suspensions to identify (1) the percentage and phenotypic expression of subsets of tumor infiltrating lymphocytes present in the tumor and (2) tumor markers, if any, which are expressed by the tumor cells. Once the tumor infiltrating lymphocyte cultures have been expanded and are to be harvested for patient therapy, we analyze the material using routine cytologic preparations and immunocytochemistry to ensure the cultures are free of tumor cells.

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

Major Findings:

Over 250 tumor suspensions have been evaluated for tumor associated antigens and for phenotypic analysis of tumor infiltrating lymphocytes including: melanomas, renal cell carcinomas and sarcomas.

Over 380 tumor infiltrating lymphocyte (TIL) cultures have been examined. Cytologically, TIL cultures consist of a monomorphic population of activated lymphoid cells resembling an immunoblastic lymphoma. The majority of reactive lymphoid cells from TIL cultures are CD3 positive with a variable proportion of cells positive for CD4 or CD8. In less than 4% of cultures, rare residual tumor cells are identified.

Publications:

Schwartzentruber DJ, Solomon D, Rosenberg SA, Topalian SL. Characterization of lymphocytes infiltrating human breast cancer: Specific immune reactivity can be detected by measuring cytokine secretion. J Immunotherapy (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09176-04 LP

PERIOD COVERED: October 1, 1991 to September 30, 1992

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

In December, 1988, the National Cancer Institute sponsored a workshop to develop uniform descriptive diagnostic terminology for cervicovaginal cytopathology to replace the Papanicolaou Class designations. The proceedings of that meeting, known as the 1988 Bethesda System, have 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 (in whole or in part), or were planning to do so in the near future. Two years after its initial publication, the time had come to critically evaluate the advantages and disadvantages of TBS in actual laboratory practice.

A "Second Conference" on The Bethesda System was held April 29 and 30, 1991, at the National Institutes of Health in Bethesda, Maryland with a capacity attendance. The two day meeting organized by the Cytopathology Section, provided open exchange of data, lively debate and a forum for critical analysis of TBS. An Editorial Committee was established to revise TBS terminology based on this input as well as written commentaries, scientific data, and laboratory surveys. The revised TBS has been significantly streamlined and simplified. A Criteria Committee is completing a reference atlas which will include morphologic criteria and accompanying photomicrographs. I have been an active participant in both committees.

In addition, I have been involved as a cytopathology resource person in numerous meetings and working groups including: The College of American Pathologist's Cytopathology Committee; Executive Committee of the American Society of Cytology; several CDC workshops on quality assurance in breast and cervical cancer; a CDC expert panel on cervical disease in HIV-infected women; a videodisc collaboration with the National Library of Medicine on cervical cancer; and the development of the National Strategic Plan for Breast and Cervical Cancer.

Publications:

Solomon D. Nomenclature for the cytodiagnosis of cervical intraepithelial lesions: Letter to the Editors. Acta Cytol 1991;35:658-9.

Solomon D. The Bethesda System: An overview. Int J Gynecol 1991;10:323-5.

Rapid Communication from the NIH: The Bethesda System for Reporting Cervical/Vaginal Cytologic Diagnoses - Report of the 1991 Bethesda Workshop. JAMA 1992;267:1892.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09363-01 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Glutathione S-transferase-pi and P-glycoprotein in FNA of Breast Tumors

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

PI:	K. Cowan	Senior Investigator	COP DCT NCI
OTHER:	D. Solomon	Chief, Cytopathology Section	LP NCI
	J. O'Shaughnessy	Medical Officer	COP DCT NCI

COOPERATING UNITS *(if any)*
DCT LP

LAB/BRANCH
Laboratory of Pathology

SECTION
Cytopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.20	.10	.10

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 glutathione S-transferases are a group of enzymes that have been shown to be active in the detoxification of certain drugs, carcinogens and metabolites. Increased expression of the placental form of the enzyme has been found in association with carcinogenesis and drug resistance. Some investigators have found increased expression of the placental form of the enzyme, measured by RNA slot blot and protein assays, to be inversely related to estrogen receptor positivity in malignant breast tumors. A study is currently underway to further evaluate this relationship using immunohistochemistry to determine GST-pi expression and estrogen receptor positivity in formalin-fixed paraffin embedded breast tumors.

We also plan to prospectively study fine needle aspirates of breast cancer patients to assess P-glycoprotein expression before and after chemotherapy to determine if a relationship exists between P-glycoprotein expression and response to therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09187-03 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Transforming Growth Factor (TGF)-ß in the Differentiation of Neuroblastoma

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

PI:	M. Tsokos	Chief, Ultrastructural Pathology Section	LP NCI
OTHER:	B. McCune	Biotechnology Fellow	LC NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	4/4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

D

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

In a recent immunohistochemical study we found lack of expression of TGF-ß1, 2, and 3 by neuroblastoma cells, except for well differentiated ganglion cells in ganglioneuroblastomas and in normal ganglia. These data suggested a possible role of TGF-ß in the differentiation of human neuroblastoma. This hypothesis will be further pursued in the following ways: (1) TGF-ß expression and secretion will be studied in neuroblastoma cell lines before and after differentiation with known differentiating agents, such as retinoic acid and TPA. (2) Direct effects of exogenously added TGF-ß in undifferentiated and differentiated neuroblastoma cell cultures will be determined. (3) Blocking antibodies and antisense TGF-ß oligonucleotides will be used to modulate possible actions of TGF-ß on neuroblastoma cells *in vitro*. Preliminary data have shown induction of TGF-ß1 mRNA in SH-SY5Y neuroblastoma cells following treatment of cultures with TPA or retinoic acid, both of which promoted differentiation. In contrast, another neuroblastoma cell line (IMR-32) which showed minimal morphologic differentiation with the same agents, showed only mild increase in TGF-ß1 mRNA levels. The differences in the TGF-ß expression by neuroblastoma cells before and after differentiation may be the result of induction of surface receptors by the differentiation agents and secondary synthesis of TGF-ß, a phenomenon which will be further pursued by binding studies to determine possible changes of TGF-ß receptors on the surface of neuroblastoma cells before and after treatment with differentiating agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09354-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Expression 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 Fogarty Fellow	LP NCI
	B. McCune	Biotechnology Fellow	LP NCI
	T. Szentendrei	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We found that rhabdomyosarcomas show consistently high levels of TGF-ß1, and to a lesser extent TGF-ß3, but lack TGF-ß2 protein by immunohistochemical staining *in vivo*. We also detected variable levels of TGF-ß protein synthesis in conditioned media of cultured rhabdomyosarcoma (RMS) cells with the mink lung fibroblast (CCL-64) bioassay and the ELISA method. Furthermore, all RMS expressed variable levels of TGF-ß1 mRNA *in vitro*. These data, in combination with our previous data of TGF-ß-induced inhibition of RMS cell differentiation *in vitro*, and the known inhibitory effect of TGF-ß in normal myogenesis have suggested to us a role of TGF-ß in human RMS. We have studied levels of TGF-ß1 mRNA expression in RMS cell lines after treatment with 5-azacytidine (AZA), an agent that causes differentiation and inhibition of cell growth in RMS, and found them increased in 2 RMS lines. We then decided to study if there were similar changes with other agents that are known to cause differentiation and growth inhibition, or growth inhibition alone, and selected one RMS cell line (RD) and 3 additional agents, i.e., retinoic acid, TPA, and cytosine arabinoside. The possible role of TGF-ß in the growth and differentiation of this RMS cell line will be pursued in the following ways: (1) Levels of expression and affinity of TGF-ß for its receptors will be studied before and after treatment with the above agents, by binding assays and PAGE of cell lysates incubated with 125I-TGF-ß1. (2) Anti-TGF-ß blocking antibodies will be used along with the agents to determine if their action is linked to levels of TGF-ß1. (3) Nuclear transcription assays will be performed to evaluate if the elevated TGF-ß1 mRNA levels are due to increased transcription rather than decreased turnover. (4) TGF-ß protein levels will be evaluated to determine if they parallel the levels of mRNA.

Publications:

McCune BK, Patterson K, Chandra RS, Kapur S, Sporn M, Tsokos M. Expression of transforming growth factor-ß isoforms in small round cell tumors of childhood: An immunohistochemical study. Am J Pathol (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09355-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Immunohistochemical Detection of Wild & Mutant Type p53 Gene 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:	A. Karameris	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

C. Felix, Assistant Professor, Children's Hospital, PA

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

CHECK APPROPRIATE BOX(ES)

☐ (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.)*

p53 protein is a 53 kd nuclear phosphoprotein, encoded by the p53 gene, a tumor suppressor gene acting probably as a negative regulator of cell growth. The single copy of the human p53 gene is on chromosome 17p and loss of homozygosity in this locus is frequently found in common human cancers such as breast, colon, small lung cell carcinoma and astrocytoma. In addition, recent findings strongly suggest that point mutations and altered expression of p53 is the most frequent known genetic change in human cancer. In collaboration with Dr. C. Felix *et al.*, we have recently shown the existence of p53 mutations in a significant proportion of cases of childhood rhabdomyosarcoma. There is evidence to support that the mechanism by which mutant p53 gene affects tumor growth is through its mutant p53 proteins whose half-lives are markedly increased as a result of formed complexes with the heat shock proteins. In this way, the levels of these mutant p53 proteins are comparable to those known to exert a "trans-dominant loss of function" effect on wild type p53 protein, which is found decreased in tumors expressing p53 gene mutations. Immunohistochemical studies of breast and colon carcinomas using antibodies against p53 protein have shown association of these proteins with high grade tumors and a potential prognostic role of this protein in human malignancy. Antibodies specific to wild or mutant p53 proteins have now become commercially available. We will use these antibodies to stain paraffin-embedded rhabdomyosarcomas of the alveolar and embryonal histologic subtypes before and after disease progression, and will compare p53 expression with histologic subtype, clinical stage and outcome. This study will help us answer the question if there is a potential role of the p53 gene in the development and prognosis of childhood rhabdomyosarcoma.

GPO 914-918

Publications:

Felix CA, Chavez Kappel C, Mitsudomi T, Nau MM, Tsokos M, Crouch GD, Nisen PD, Winick NJ, Helman LJ. Frequency and diversity of p53 mutations in childhood rhabdomyosarcoma. Cancer Res 1992;52:2243-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09370-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Detection of p53 Mutations in Ewing's Sarcoma and 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:	A. Karameris	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

p53 mutations have been detected in many tumors and interpreted as a late event in tumorigenesis, probably involved in tumor progression rather than tumor development. Recent studies have demonstrated the presence of germline p53 mutations in some Li-Fraumeni pedigrees and hence a role of p53 mutations in hereditary susceptibility to human cancer. Rhabdomyosarcoma, a common soft tissue sarcoma in the families with the Li-Fraumeni syndrome, was found to have p53 mutations. However, the frequency of such mutations in pediatric tumors in general remains unknown. We will study the presence of p53 mutations in established Ewing's sarcoma cell lines in comparison with existing data from rhabdomyosarcoma cell lines. In addition, p53 mutations will be studied in paraffin embedded tissues from rhabdomyosarcoma tumors to investigate the validity of paraffin embedded tissues for such studies. The detection of mutations will be performed on PCR fragments spanning exons 4, 5, 6, 7, and 8 of the p53 gene. Two methods will be compared: the standard single stranded conformational polymorphism (SSCP) method, and a non-radioactive method, based on conformational differences in double stranded molecules (heteroduplex analysis) and using non-denaturing electrophoresis in mutation detection enhancement (MDE) gels.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09371-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Muscle Determination Genes in the Diagnosis of Childhood 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:	A. Karameris	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	4/4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Although distinction of rhabdomyosarcoma from the other round cell tumors of the soft tissues in children is not difficult in most cases, the argument as to what connotes a primitive rhabdomyosarcoma and an extraskeletal Ewing's sarcoma is a valid one. Furthermore, this argument comprises more than a philosophical question, since patients with rhabdomyosarcoma are treated in a different protocol than those with Ewing's sarcoma. We have recently studied immunohistochemically the presence of the product of the pseudoautosomal MIC2 gene in a series of childhood tumors and found preferential expression of this product by Ewing's sarcoma and lack thereof in rhabdomyosarcoma. However, a few primitive tumors expressing 1 or 2 muscle markers (i.e. skeletal actin and/or desmin) and ultrastructural appearance favoring a primitive rhabdomyosarcoma, were found positive for the MIC2 product, while others with similar characteristics were negative. Since several muscle determination genes have recently been cloned and cDNA probes have become commercially available, we decided to study the expression of these genes in the same cases that have been studied by the MIC2 gene product and find out if these markers are more reliable of myogenesis than the MIC2 gene product or the existing antibodies against muscle proteins. For this study, DNA will be extracted from 50 micron sections of paraffin-embedded tissues and will be analyzed by Southern blotting for the presence of MyoD1, Myogenin, Myf5, Myf6, skeletal myosin heavy chain and muscle creatine kinase. The value of these markers over the more traditional ones will be critically evaluated, expecially in those primitive cases that are difficult to distinguish because of conflicting immunohistochemical data.

GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09172-04 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

PI:	D. Roberts	Chief, Biochemical Pathology Section	LP NCI
OTHER:	V. Zabrenetzky	Staff Fellow	LP NCI
	N. Guo	Visiting Associate	LP NCI
	T. Vogel	General Fellow	LP NCI
	E. Negre	Visiting Fellow	LP NCI

COOPERATING UNITS *(if any)*

Howard Fillit, Mount Sinai Medical Center; Patricia Steeg, Lab. of Path., NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	3.0	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The functions of thrombospondin in cell adhesion and migration and tumor metastasis are being investigated. We have identified two regions of the thrombospondin molecule that mediate adhesive and migratory responses of cultured human melanoma cells to thrombospondin. The carboxyl-terminal domain mediates attachment and haptotaxis, while the amino-terminal domain mediates cell spreading and chemotaxis. The cell receptors recognizing these two regions of thrombospondin are under investigation. One class of receptors are sulfated glycoconjugates that bind to the amino-terminal domain of thrombospondin. A minor heparan sulfate proteoglycan that binds thrombospondin with high affinity was identified in two melanoma cell lines (Cancer Res 48;1988:6875). An unusual sulfated glycolipid present only in melanoma cell lines that spread on thrombospondin binds to thrombospondin and mediates melanoma cell spreading (ibid.). To further define the mechanism of thrombospondin interactions with tumor cells, receptors for the carboxyl-terminus of thrombospondin are being characterized. Synthetic peptides from this region of thrombospondin are being used to define the sites recognized by thrombospondin receptors. The intracellular responses of cells to binding of thrombospondin to the two types of receptors are also being investigated. Expression of thrombospondin mRNA in tumor cells is being examined to look for association of thrombospondin synthesis with *in vivo* metastatic potential.

GPO 914-918

Major Findings:

1) Peptides from the three type I repeats of human endothelial cell thrombospondin, containing the consensus sequence - Trp-Ser-Xaa-Trp-, bind to sulfated glycoconjugates including heparin and sulfatide. The peptides are potent inhibitors for the binding of thrombospondin, laminin, or apolipoprotein E to these ligands. The thrombospondin peptides which inhibit heparin binding, but not adjacent peptides from the thrombospondin sequence containing the previously identified adhesive motif Val-Thr-Cys-Gly, promote melanoma cell adhesion when immobilized on plastic. Melanoma cell adhesion to the immobilized peptides is inhibited by soluble recombinant heparin-binding fragment of thrombospondin. The peptides also inhibit heparin-dependent binding of thrombospondon or laminin to human melanoma cells. The active peptides lack any previously identified heparin-binding consensus sequences and most do not contain any basic amino acids. Studies with homologous peptides showed that the tryptophan residues are required for binding. Adjacent basic residues in the second type I repeat enhance binding to heparin but not to sulfatide. The type I peptides of thrombospondin thus define a new class of heparin-binding peptides.

Further studies using these peptides demonstrated that the two Trp residues and the Ser residue are essential. The Trp residues must be spaced less than four residues apart. The Pro residue is essential for activity of the pentapeptide Trp-Ser-Pro-Trp-Ser, but some larger peptides with substitutions of the Pro residue are active. Peptides containing the consensus sequence and basic amino acids are chemotactic for A2058 human melanoma cells. The functional importance of this novel heparin and sulfatide binding motif is suggested by its conservation in other members of the thrombospondin gene family, complement components, and in many members of the cytokine receptor and transforming growth factor ß superfamilies.

2) Tumor cell TSP steady state mRNA levels are inversely related to malignant progression. TSP mRNA levels were determined by hybridization of TSP cDNA probes on Northern blots. TSP mRNA levels were compared to the tumorigenic and metastatic potential of tumor cell lines determined by s.c. or i.v. injection into Nu/Nu mice. In the K-1735 murine melanoma, the low metastatic Clone 19 cell line contained high levels of TSP mRNA levels and the high metastatic TK cell line had none. The highly metastatic MDA-MB-435 human breast cell line had only one third the level of TSP mRNA expressed in the non-metastatic and estrogen-dependent MCF-7 cell line. An immortalized human bronchial epithelial cell line (BEAS-2B) contained higher levels of TSP mRNA levels compared to three ras transformants. Cell lines derived from subcutaneous tumors of each ras transformant, which exhibited increased tumorigenic and metastatic behavior *in vivo*, contained further reductions in TSP mRNA levels. Expression of the extracellular matrix molecule fibronectin was not correlated with malignant progression in these cell lines. Thus, in a variety of tumor cell types, malignant progression is specifically associated with reduced expression of TSP mRNA.

3) Murine EHS tumor laminin and human platelet thrombospondin bound with high affinity to a vascular heparan sulfate proteoglycan purified from bovine kidney.

Binding of thrombospondin was comparable to the isolated heparan sulfate chains and the intact proteoglycan and did not require the core protein. Thrombospondin binding to the intact proteoglycan, heparan sulfate, or heparin-bovine serum albumin immobilized on plastic was heterogeneous with at least two classes of binding affinities. Recombinant heparin-binding domain from thrombospondin also bound specifically to the proteoglycan, heparin-BSA, and sulfatide. Binding of laminin to the HSPG or heparin at 2°C could be described by a single class of high affinity binding sites. At higher temperatures, however, the Scatchard plots were concave downward. Temperature-dependent apparent cooperative binding was also observed for laminin binding to sulfatide. The binding data could not be fit by a positive cooperative binding model, but probably results from ligand induced oligomerization of the laminin at higher temperatures. Approximately 60% of thrombospondin or laminin binding to cultured bovine aortic endothelial cells was heparin dependent. The cells expressed proteoglycans that bound thrombospondin and laminin and cross reacted with a monoclonal antibody to the core protein of the kidney proteoglycan.

Publications:

Guo N, Krutzsch HC, Negre E, Vogel T, Blake DA, Roberts, DD. Heparin- and sulfatide-binding peptides from the type I repeats of thrombospondin promote melanoma cell adhesion. Proc Natl Acad Sci USA 1992;89:3040-4.

Roberts DD. Interactions of thrombospondin with sulfatides and other sulfated glycoconjugates. In: Lahav J, ed. Thrombospondin. CRC Press (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09173-04 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Carbohydrate Receptors for Human Pathogens

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

PI:	D. Roberts	Chief, Biochemical Pathology Section	LP NCI
OTHER:	E. Negre	Visiting Fellow	LP NCI

COOPERATING UNITS *(if any)*

T. Walsh, Pediatric Oncology, NCI, NIH; R. Mecham, Washington University Medical Center, St. Louis, MO

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Adhesive specificities of *Staphylococcus aureus*, some *Enterocuccus* species, *Candida albicans*, and elementary bodies of *Chlamydia trachomatis* are being examined. These have been screened for binding to glycoproteins and glycolipids of known structure and to glycoconjugates isolated from target tissues to which the pathogens adhere. Where possible, inhibitors of each binding specificity will be identified using the solid phase assays and then tested using *in vitro* cytoadherence assays and *in vivo* infection assays to determine the role of each in cytoadherence and initiation of infection.

Major Findings:

Human tropoelastin binds specifically to *S. aureus*. Binding specificity was shown by competition assays in which binding of radiolabeled tropoelastin was inhibited by elastin peptides but not be control proteins or peptides. Binding is of high affinity (4-7 nM) to approximately 1100 sites per organism. Binding is protease sensitive and requires the 30 kDa amino-terminal fragment of tropoelastin. Binding is specific to *Staphylococci* species known to infect elastin-rich sites *in vivo*.

We are studying the binding of fibronectin and recombinant and proteolytic fragments of this protein to *Candida albicans* to determine which of the numerous functional domains of the protein are involved in the interactions between the yeast and the protein. *In vitro* assays were developed to quantify the attachment of *C. albicans* to fibronectin or the fragments coated on an insoluble synthetic matrix, and to evaluate the binding of soluble fibronectin and the fragments to *C. albicans* in suspension. *C. albicans* culture has been the critical step to obtain reproductive results. Expression of the receptor depends on growth medium, temperature, shear, and time in culture. Optimal conditions have been established to obtain high receptor expression. Binding of labeled fibronectin to *C. albicans* is saturable and reversible but requires an unusual 3 hours to teach equilibrium. 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.

Publications:

Park PW, Roberts DD, Grosso LE, Parks WC, Rosenbloom J, Abrams WR, Mecham RP. Binding of tropoelastin to *Staphylococcus aureus*. J Biol Chem 1991;266:23399-23406.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09174-04 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Role of Sulfated Glycoconjugates in Tumor Cell Adhesion

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

PI:	D. Roberts	Chief, Biochemical Pathology Section	LP NCI
OTHER:	N. Guo	Visiting Fellow	LP NCI
	T. Vogel	General Fellow	LP NCI
	H. Yu	Visiting Associate	LP NCI

COOPERATING UNITS *(if any)*

H. Gralnick, Hematology Service, CC, NIH; J. Cashel, Biochemical Pathology Section, Lab. of Path., NCI; H. Fillit, Mount Sinai Medical Center

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have found that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion (Cancer Res 1988;48:3367) and as receptors for thrombospondin on melanoma cells (Cancer Res 1988;48:6785). Relatively few of the glycolipids belonging to this class have been characterized. We are purifying novel sulfated glycolipids from several sources including human kidney and meconium and breast and small cell lung carcinoma cell lines. The structures of these glycolipids will be examined using chemical and immunological approaches. Monoclonal antibodies to these will be used to examine the potential of these structures as tumor markers. The molecular basis of binding of adhesive glycoproteins to sulfatide and heparan sulfate proteoglycans are being investigated by identifying sequences in these molecules responsible for binding. Both proteolytic and recombinant fragments of the adhesive proteins are being used to map the active binding domains.

Major Findings:

1. Unstimulated human platelets from normal volunteers adhere to sulfatides but not to other glycolipids or phospholipids. Binding is saturable and dose-dependent. Platelets from a patient with severe Type I von Willebrand's disease adhere poorly to sulfatides. However, adhesion to levels seen with normal platelets is retored by the addition of von Willebrand factor. Adhesion of normal platelets can be partially inhibited by a monospecific antibody to vWf. Both vWf binding and platelet adhesion to sulfatide are inhibited by dextran sulfate but not by heparin, fibrinogen, fibronectin, or RGD-peptides. Thus, adhesion to sulfatides is of two types: vWf-dependent (50-75%) and vWf-independent (25-50%).

2. A laminin-binding peptide (peptide G), predicted from the cDNA sequence for a 33 kDa protein related to the 67 kDa laminin receptor, specifically inhibits binding of laminin to heparin and sulfatide. Since the peptide binds directly to heparin and inhibits interaction of another heparin-binding protein with the same sulfated ligands, this inhibition is due to direct competition for binding to sulfated glycoconjugates rather than an indirect effect of interaction with the binding site on laminin for the 67 kDa receptor. Direct binding of laminin to the peptide is also inhibited by heparin. This interaction may result from contamination of the laminin with heparan sulfate, as binding is enhanced by addition of substoichiometric amounts of heparin but inhibited by excess heparin and two heparin-binding proteins. Furthermore, laminin binds more avidly to a heparin-binding peptide derived from thrombospondin than to the putative receptor peptide. Adhesion of A2058 melanoma cells on immobilized peptide G is also heparin-dependent, whereas adhesion of the cells on laminin is not. Antibodies to the ß1 integrin chain or laminin block adhesion of the melanoma cells to laminin but not to peptide G. Thus, the reported inhibition of melanoma cell adhesion to endothelial cells by peptide G may result from inhibition of binding of laminin or other proteins to sulfated glycoconjugate receptors rather than from specific inhibition of laminin binding to the 67 kDa receptor.

Publications:

Data RE, Williams SB, Roberts DD, Gralnick HR. Platelets adhere to sulfatides by von Willebrand factor dependent and independent mechanisms. Thromb Haem 1991;65:581-7.

Guo N, Krutzsch HC, Vogel T, Roberts DD. Interactions of a laminin-binding peptide from a 33-kDa protein related to the 67-kDa laminin receptor with laminin and melanoma cells are heparin-dependent. J Biol Chem (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09175-04 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Glycoconjugate Antigens Expressed in Cancer Cells

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

PI:	D. Roberts	Chief, Biochemical Pathology Section	LP NCI
OTHER:	H. Yu	Visiting Associate	LP NCI

COOPERATING UNITS *(if any)*

R. Goldblum, Department of Pediatrics, University of Texas Medical Branch, Galveston; S. Yedgar, Hebrew University, Jerusalem

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Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Altered glycosylation of glycolipids and glycoproteins is frequently observed in tumor tissues and cultured cancer cell lines. Changes in expression of glycosyltransferases produce unique carbohydrate structures which can be used to differentiate tumor from normal tissue and in some cases alter the biological activity of adhesive proteins and receptors which bear the altered oligosaccharides. We are examining the specificity of some monoclonal antibodies that recognize oligosaccharide determinants on functionally important molecules and the structures of sulfated glycoconjugates produced by cancer cells which bind to adhesive proteins such as laminin and thrombospondin. Currently we are examining globo-series sulfated glycolipids produced in human breast cancer and carbohydrates recognized by monoclonal antibodies to human secretory component and sialyl Lewis a which recognize glycoproteins and glycolipids produced in colon adenocarcinomas.

Major Findings:

1. Antibody 6C4 is a mouse IgM monoclonal antibody against human secretory component that also binds to some colon adenocarcinoma cell lines including HT29 (Woodward *et al.*, J Immunol 1984;133:2116-2125), LS-180 and SW1116 cells. Asparagine-linked oligosaccharides contain the epitope recognized by this antibody. Inhibition of antibody binding to free secretory component by human milk oligosaccharides established that lacto-N-tetraose is the minimum structure recognized by the antibody, but larger oligosaccharides with terminal type I saequences bind with much higher affinity. The antibody also binds to type I oligosaccharide sequences substituted with Fucα1-4GlcNAc but not with Fucα1-2Gal. Milk oligosaccharides containing the Lewis Fucα1-4GlcNAc bind with higher affinity than those lacking fucose. However, free secretory component does not bind antibodies to Le^a or Le^b oligosaccharides, and the Le^a antibody does not inhibit 6C4 binding to free secretory component. Therefore, the epitope recognized by 6C4 on free secretory component is not an asparagine-linked Le^a oligosaccharide. A monoclonal antibody affinity column was prepared and used to isolate oligosaccharides from human milk, free secretory component, and human lactoferrin that bind the antibody. Oligosaccharides were obtained that bind to the column at 4° and are eluted by increasing the temperature to 37°. The structures of these oligosaccharides are being determined.

2. The monoclonal antibody 19-9 recognizes carbohydrate antigens on glycolipids and mucins produced by adenocarcinomas. SW-1116 colon adenocarcinoma cells constitutively secrete mucin containing this epitope. Secretion is independent of cAMP level but can be stimulated by the Ca ionophore A23187. Arachidonic acid and its metabolites inhibit secretion. Electron microscopic studies reveal mucin near the plasma membrane and in vesicular structures. The control of secretion of mucin by SW1116 cells is relevant to pathological states associated with excessive constitutive secretion of mucin including gastrointestinal cancer and cystic fibrosis.

Publications:

Yedgar S, Eidelman O, Molden E, Roberts DD, Etcheberrigaray R, Goping G, Pollard HB. cAMP-independent secretion of mucin by SW1116 human colon carcinoma cells: differential control by calcium ionophore A23187 and arachidonic acid. Biochem J (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00891-09 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
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
	J. Murata	Visiting Fellow	LP NCI
	Å. Arestad	Special Volunteer	LP NCI
	H. Krutzsch	Research Chemist	LP NCI

COOPERATING UNITS *(if any)* R. Hopkins and P. Harley, PRI/Dyne Corp., FCRDC; M. Sveda, OTC/Biotechnology Research Division, Gaithersburg, MD; K. Williams and K. Stone, W.M. Keck Foundation, Biotechnology Resource Lab., New Haven, CT; C. Ming, Molecular Oncology Institute, Gaithersburg, MD

LAB/BRANCH
Laboratory of Pathology

SECTION
Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.8	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard 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 120,000 daltons and a pl ~ 7.8. It stimulates motility in tumor cells when present at concentrations in the picomolar to nanomolar range; this stimulation is pertussis toxin sensitive. The protein appears to be N-terminal blocked; therefore, sequence information was obtained by partially digesting purified ATX and sequencing 19 of the resultant peptides. Anti-peptide antibodies, which recognize the protein in immunoblots, have been produced in rabbits. Oligonucleotides have been synthesized which correspond to the peptide sequences and have been used both as primers in polymerase chain reaction (PCR) amplifications and as probes to screen cDNA libraries made from the same melanoma cell line. In addition, we are continuing to characterize the protein and its active site by studies with endoglycosidases to identify the nature of the linked sugar moieties.

Major Findings:

I. Purification and Partial Sequence Analysis of Autotaxin (ATX)

This 120,000 dalton protein has been purified to homogeneity and partially sequenced. Approximately 200 L of A2058 serum-free conditioned medium was prepared by the Frederick Cancer Research Facility and concentrated to ~ 8L using ultrafiltration membranes that have very low protein binding capacity. We further concentrated this material, then purified the protein using a series of chromatographic separations: phenyl sepharose (hydrophobic interaction) chromatography, concanavalin A lectin affinity chromatography, weak anion exchange chromatography, molecular sieves, and strong anion exchange chromatography. After these chromatographic procedures, a single 120 kDa protein band remained associated with motility-stimulating activity. Alternately, the partially purified ATX, which had not undergone the final separation step on strong anion exchange chromatography, could be separated on a polyacrylamide SDS gel and electroeluted out of the gel to give a single protein band. This purified protein was then digested by cyanogen bromide followed by trypsin. The resultant peptides were purified on reverse phase chromatography, and subjected to micro-Edman degradation. Using these techniques, sequence information was obtained on 19 peptides.

II. Anti-Peptide Antibodies

Several peptide sequences from ATX were synthesized in large quantities, cross-linked to bovine serum albumin, and used to immunize rabbits. Anti-serum was purified by ammonium sulfate precipitation followed by peptide affinity chromatography. Two of these purified antibodies were found to bind to a single 120 kDa protein on immunoblots. To date, the antibodies neither immunoprecipitate nor neutralize activity. Three sets of rabbits are in the early stages of immunization and have not yet been tested.

III. Plans for Future Study

The primary goal for the immediate future is to clone the gene for ATX. Because ATX is made by the cells in small quantities and because the mRNA for ATX appears to be rare on northern blots, we are attempting to achieve this goal by using a number of complementary approaches. Oligonucleotides have been synthesized which are derived from the peptide amino acid sequence. These oligonucleotides are being used directly to probe cDNA libraries derived from A2058 cells. In addition, the oligonucleotides can serve as primers for both reverse transcriptase reactions using total cellular RNA as template and for PCR amplification using cDNA as template. The amplified DNA sequences obtained by these methods, presumed to contain partial gene sequence, will be used as alternative probes of the cDNA library. Finally, the anti-peptide antibodies, which are very potent on immunoblots, can be used as secondary probes of our expression library.

We plan to continue with the rabbit antipeptide studies. In addition, we plan to immunize mice with preparations containing either mixtures of the conjugated peptides or the whole AMF molecule. We will screen these antibodies using assays to determine direct neutralization of activity, immunoprecipitation of activity, and Western blotting of appropriate bands. We will also utilize these antibodies to screen a cDNA expression library and to perform immunohistochemical stains of normal and tumor tissue.

Finally, we will continue to produce conditioned medium and to purify ATX. This material will be used for several ongoing projects. First, it will be used to immunize mice for monoclonal antibodies. Second, it will be used for studies with endoglycosidases and neuraminidase to determine whether N-linked or O-linked sugars are part of ATX. We will also attempt to assess the role of these sugars in cellular activation. Finally, we will radiolabel the pure protein and begin to characterize the AMF receptor. The sensitivity of the AMF-stimulated response to pertussis toxin suggests that AMF works through a cell surface receptor and that the signal is transduced across the plasma membrane through a G protein. Using simple binding assays and cross-linking procedures, we can begin to determine the size and nature of the AMF receptor.

Publications:

Stracke ML, Krutzsch HC, Unsworth EJ, Årestad A, Cioce V, Schiffmann E, Liotta LA. Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. J Biol Chem 1992;267:2524-9.

Boike G, Lah T, Sloane BF, Rozhin J, Honn K, Guirguis R, Stracke ML, Liotta LA, Schiffmann E. A possible role for cysteine proteinase and its inhibitors in motility of malignant melanoma and other tumour cells. J Melanoma Res 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00892-09 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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	Senior Staff Fellow	LP NCI
OTHER:	N. MacDonald	Visiting Fellow	LP NCI
	A. Leone	Visiting Associate	LP NCI
	U. Flatow	Biologist	LP NCI
	M. Benedict	Biologist	LP NCI
	A. De La Rosa	Visiting Fellow	LP NCI
	D. Weinstat-Saslow	Staff Fellow	LP NCI

COOPERATING UNITS *(if any)*

Molecular Oncology, Inc., Gaithersburg, MD (CRADA);
R. Callahan, LTIB, DCBDC, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.1	4.1	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.)*

Multiple projects are underway to investigate the molecular mechanisms of cancer progression, with research emphasis on women's cancers. The role of the nm23 family of genes in the regulation of tumor metastasis was investigated. Reduced expression of nm23 protein levels in highly metastatic breast carcinomas was observed in two immunohistochemistry studies, and somatic allelic deletion of nm23-H1 was found to contribute to its reduced protein expression. Transfection of the human nm23-H1 cDNA into the metastatic human breast carcinoma cell line MDA-MB-435 is near completion. The nm23-H1 transfectants exhibited reduced activity upon growth *in vitro*, colonization in soft agar, responsiveness to TGF-B in colonization, and migration to serum, PDGF, and IGF in chemotaxis assays, as compared to control transfectants. Upon injection into the mammary fat pad, all lines formed primary mammary tumors, but the nm23-H1 transfectants produced metastases to the draining lymph node and/or lungs in significantly fewer mice. The data indicate that expression of the human nm23-H1 cDNA can suppress the malignant progression of this human breast carcinoma cell line. Transfection experiments using a human ovarian carcinoma cell line are underway.

For both the murine K-1735 TK melanoma and human MDA-MB-435 breast carcinoma cell lines, nm23-H1 transfectants were more sensitive to inhibition of *in vitro* growth by the chemotherapeutic drug cisplatin than control transfectants. In the murine melanomas, nm23 transfectants also exhibited reduced levels of a protein thought to be part of the DNA repair complex, XPE-BF. The data suggest an unexpected association between suppressor gene expression and sensitivity to DNA damaging agents.

Additional research has investigated the expression of the extracellular matrix protein thrombospondin (TSP) in cancer progression. Highly tumorigenic and/or metastatic cell lines from murine melanomas, human lung carcinomas and human breast carcinomas expressed quantitatively reduced TSP, as compared to less aggressive controls. The TSP cDNA has been subcloned into an expression construct, in anticipation of transfection experiments in human breast carcinoma cell lines. Molecular investigations are underway to evaluate the growth factor receptor and cyclin expression of premalignant lesions of the human breast.

Major Findings:

Research in the past year has emphasized molecular alterations in women's cancers. The major research project concerned nm23, a metastasis suppressor gene. Additional research projects are investigating the expression and function of thrombospondin, a putative angiogenesis inhibitor, and the molecular basis for atypical ductal hyperplasias of the human breast.

nm23 and Tumor Metastasis: The nm23 family of genes was discovered in my laboratory by differential hybridization between low and high metastatic potential murine melanoma cell lines. Transfection of the murine nm23-1 cDNA into murine K-1735 TK melanoma cells resulted in reduced primary tumor incidence, significant reductions in metastatic potential, and altered responsiveness *in vitro* to TGF-ß, establishing nm23 as a suppressor gene.

For breast cancer, low nm23 RNA and/or protein expression has been associated with lymph node metastases, disease recurrence, and poor survival in four studies from three independent laboratories. We published the first study of nm23 protein expression, determined by immunohistochemistry. Diffuse or focal low nm23 protein expression, determined by three pathologists, was significantly associated with reduced patient overall survival. A second immunoperoxidase staining study confirmed and extended these findings: In collaboration with Drs. Robert Callahan (NCI) and Rosette Lidereau (Centre Rene Hugenin, St. Cloud, France), breast carcinoma nm23-H1 protein expression was compared to patient metastasis-free survival. Focal or diffuse low nm23-H1 protein staining in tumor sections was again associated with reduced patient metastasis-free survival, determined independently by two pathologists. Diffuse low nm23-H1 expression was associated with nm23-H1 somatic allelic deletion in 83% of the cases examined, implicating this genetic alteration in the regulation of nm23 expression. No evidence of mutations in the translated sequence of nm23-H1 was observed in 20 tumors. Current efforts are identifying and characterizing the nm23-H1 and nm23-H2 genomic sequences, to determine if mutations exist in the regulatory portions of these genes in breast cancer. The 3' portions of the nm23-H1 and nm23-H2 have been identified, as well as a nm23-H2-like pseudogene.

Transfection of the human nm23-H1 gene into the human MDA-MB-435 breast carcinoma cell line is near completion. MDA-MB-435 cells were transfected with a pCMVneo expression construct containing the full length nm23-H1 cDNA and two stable, high expressing clones selected (H1-170, H1-177). As controls, MDA-MB-435 cells were transfected with the empty pCMVneo construct, and two clones randomly selected (C-100, C-103). *In vitro* assays of growth and metastatic properties indicated significant differences between the control and nm23-H1 transfectants: These include a reduced growth rate at low passage number, reduced colonization potential in soft agar, altered responsiveness to TGF-B, and reduced migration to serum, PDGF, IHG, and an autocrine motility factor (AMF). Upon injection of control and nm23-H1-transfected clones into the mammary fat pad of nude mice, the mean primary tumor size of the nm23-H1 transfectants was smaller than that of the control transfectants only for the highest nm23-H1-transfected line, at low passage number. The discordance of *in vitro* growth and colonization assay data with primary tumor size *in vivo* indicate the importance of an orthotopic injection site, which may provide tumor-stromal interactions and/or local growth factors not available *in vitro*. Both the H1-170 and H1-177 clones produced metastases to the draining lymph nodes and lungs in fewer animals than did the control transfected clones. The data indicate a negative regulatory effect of nm23-H1 expression on MDA-MB-435 breast carcinoma malignant progression. We propose the use of the MDA-MB-435 system in the NCI's drug screening program, and anticipate efforts to identify

drugs and/or cytokines which stimulate breast carcinoma cell nm23-H1 expression. These studies were conducted in collaboration with Drs. Judah Folkman and Bruce Zetter (Harvard) and the staff of the animal facility at the Frederick Cancer Research Facility.

Studies of the role of nm23 in the normal development and differentiation of the mouse mammary gland have been conducted in collaboration with Dr. Sandra Haslam (Michigan State University). Nm23 protein levels were low in the immature, proliferating mammary epithelial cells, and increased in the mature nulliparous mammary gland. Nm23 expression was down-regulated by estrogen, but not progesterone in experiments where mice were ovarectomized and given exogenous hormones. Additional developmental studies are underway in collaboration with Drs. Heiner Wesphal (NIH) and David Burstein (NYU).

An additional transfection experiment is underway using the human OVCAR-3 ovarian carcinoma cell line. Stable clones which were transfected with a control, nm23-H1, or nm23-H2 constructs have been identified, and are under characterization.

In collaboration with Drs. Dennis Slamon (UCLA) and Robert Seeger (USC), we have identified a novel series of molecular alterations to the nm23 genes in childhood neuroblastoma. Using a cohort of 75 tumors, high nm23 RNA expression significantly correlated with reduced patient disease-free survival in the overall and N-myc nonamplified portion of the cohort. This pattern was the exact reverse of that observed in breast carcinoma. Overexpression of nm23 in neuroblastoma was found to result from amplification and mutation of the genes. One mutation confirmed by sequence analysis was in the putative leucine zipper motif of nm23-H2, suggesting the potential importance of this motif in nm23 function. The data provide evidence that alterations to nm23, other than its reduced expression, can be associated with high metastatic potential and provide the first evidence for nm23 mutations in a human cancer.

The potential biochemical mechanism of nm23 action is under investigation. The nm23 proteins have nucleoside diphosphate kinase (NDPK) activity. However, transfection of the murine nm23-1 cDNA into the murine K-1735 TK melanoma cell line resulted in only 10% increases in total NDPK activity. Site-directed mutagenesis experiments are underway to ablate the NDPK activity and determine whether the mutated nm23-1 cDNA will suppress TK cell metastasis upon transfection. Similar experiments will also remove the leucine zipper motif, which was the site of mutation in a Stage IV neuroblastoma. We have also found that nm23 proteins, both bacterially expressed and immunoprecipitated from breast carcinoma cell lines, autophosphorylate *in vitro*. Autophosphorylation was determined to include, in part, a serine residue, indicated by its acid/base sensitivity and phosphoamino acid analysis. Orthophosphate labeling of breast carcinoma cells *in vivo* has also indicated the presence of a phosphorylated nm23 protein, with acid/base characteristics of a phosphoserine. Since the NDPK reaction has an obligate phosphohistidine intermediate, the presence of a phosphoserine residue on nm23 may identify a new biochemical activity for this protein.

nm23 and Cisplatin Inhibition of Tumor Cell Growth: The nm23-1-transfected murine K-1735 TK melanoma cells were found to express reduced levels of a component of the DNA repair complex, the amount of a XPE-BF DNA binding factor as compared to control TK transfectants. Both nm23-1 transfectants were also more sensitive to cisplatin inhibition of growth *in vitro* than control transfectants. Nm23-1-transfected K-1735 TK cells were not more sensitive to taxol, which does not act by a DNA repair mechanism. The MDA-MB-435 breast

carcinoma cell lines transfected with nm23-H1 were also more sensitive to cisplatin inhibition of cell growth than the control transfectants. The data point to an unexpected correlation of the regulation of tumorigenicity/metastasis and DNA repair.

Thrombospondin expression and function in malignant progression: In collaboration with Drs. Vivian Zabrenetzsky and David Roberts (LP, NCI) we have investigated the expression of thrombospondin (TSP) mRNA and protein biosynthetic levels in high and low metastatic potential cell lines from murine melanomas, human lung carcinomas, and human breast carcinomas. In each case we observed a quantitative reduction in TSP expression in the highly tumorigenic and/or metastatic lines. These findings support published data which suggest a role for TSP in the inhibition of angiogenesis. The TSP cDNA has been cloned into an expression vector, and transfection experiments are underway in human breast carcinoma cell lines to determine the functional consequences of its re-expression.

Molecular alterations in premalignant lesions of the breast: In collaboration with Dr. David Page (Vanderbilt), we have begun a molecular characterization of premalignant lesions of the breast. Probes for four growth factor receptors and cyclins A and D are being subcloned into pGEM vectors for use in *in situ* hybridizations. We plan to determine the expression of these genes in atypical ductal hyperplasias (ADH) of the breast, the associated normal mammary epithelia of these patients and normal mammary epithelia of reduction mammoplasty patients. The latter two tissues are especially important comparisons, as patients with ADH have a five-fold higher risk of developing a subsequent independent carcinoma, and alterations other than those in the ADH lesion itself may be evident.

Publications:

Haut M, Steeg PS, Willson JKV, Markowitz SD. Induction of nm23 gene expression in human colonic neoplasms and equal expression in colon tumors of high and low metastatic potential. J Natl Cancer Inst 1991;83:712-6.

Barnes R, Masgood S, Barker E, Rosengard AM, Coggin DL, Crowell T, King CR, Porter-Jordan K, Wargotz ES, Liotta LA, Steeg PS. Low nm23 protein expression in infiltrating ductal breast carcinomas correlates with reduced patient survival. Am J Pathol 1991;139:245-50.

Steeg PS. Tumor metastasis: Before the revolution. Cell 1991;66:835-6.

Cohn KH, Wang F, DeSoto-LaPaix F, Solomon WB, Patterson LG, Arnold MR, Weimar J, Feldman JG, Levy AT, Leone A, Steeg PS. Association of nm23-H1 allelic deletions with distant metastases in colorectal carcinoma. Lancet 1991;338:722-4.

Steeg PS, Cohn KH, Leone A. Tumor metastasis and nm23: Current concepts. Cancer Cells 1991;3:257-62.

Golden A, Benedict M, Shearn A, Kimura W, Leone A, Liotta LA, Steeg PS. Nucleoside diphosphate kinases, nm23 and tumor metastasis: Possible biochemical mechanisms. In: Benz C, Liu, eds. Oncogenes II. Kluwer Publ. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09131-08 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Role of Laminin Binding Proteins in Human Cancer

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

PI:	M. Sobel	Senior Investigator	LP NCI
OTHER:	V. Castronovo	Visiting Scientist	LP NCI
	F. van den Brule	Visiting Fellow	LP NCI
	L. Wrathall	Biologist	LP NCI
	T. Simmons	Biologist	LP NCI
	V. Bharat	Stay-in-School	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	0.9	1.4

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. We have identified, isolated, and characterized three laminin binding proteins that are present in both normal and neoplastic tissues. All three proteins share a common epitope, and specifically bind to the poly-N-acetyllactosamine chains of laminin. The 67 kDa high affinity laminin receptor (67LR) has been previously characterized as a nonintegrin binding protein and has been molecularly cloned. It is expressed to a greater degree in metastatic tissues than in benign conditions in a variety of tissue-specific neoplasms. The 67LR is synthesized from a cytoplasmic precursor with an approximate molecular mass of 37 kDa. Using synthetic peptides, we have identified a 20 amino acid region of the precursor, designated peptide G, that binds directly to laminin with high affinity and that can inhibit attachment of laminin-coated melanoma cells to endothelium. We have also identified a specific site on the laminin molecule to which the 67LR binds. We recently purified two other nonintegrin laminin binding proteins, HLBP31 and HLBP14, from human cancer cell lines. HLBP31 and HLBP14 have apparent molecular masses of 31 kDa and 14 kDa, respectively. We isolated a cDNA clone of HLBP31 and have used cDNA clones of the 67LR and the HLBP31 to assess the relative expression of mRNA in human colorectal carcinomas. The level of HLBP31 mRNA is inversely modulated with the 67LR in human colorectal carcinomas. Using the *in situ* hybridization technique, we established that the colonic cancer cells express more 67LR mRNA than do benign epithelial mucosal cells. Future studies will determine if the selective use of different laminin binding proteins by colonic cancer cells may play a functional role in the disease process.

Major Findings:

Biochemistry of the 67LR. Previous experiments demonstrated that the 67LR is synthesized from a cytoplasmic precursor with an apparent molecular mass of 37 kDa (37LRP). Using synthetic peptides and anti-synthetic peptide antibodies, we identified a 20 amino acid domain (peptide G) that binds directly to laminin with high affinity. Peptide G can inhibit the attachment of laminin-coated melanoma cells to endothelium, thus inhibiting a critical step of the metastatic cascade.

Binding site on laminin for the 67LR. Laminin is composed of three chains that have been visualized by rotary shadowing as a cross. We have demonstrated that purified 67LR binds to laminin at a specific site, on the long arm just below the intersection of the cross. This was verified by affinity chromatography experiments in which 67LR bound to laminin fragments containing the long arm but did not bind to short arm laminin fragments.

Identification of two additional nonintegrin laminin binding proteins. We identified and purified two additional laminin binding proteins, designated HLBP31 and HLBP14, from both human cancer cell lines and from human placenta. The proteins were purified by electroelution, digested with cyanogen bromide and trypsin, and the generated peptides were microsequenced. HLBP31, with a molecular mass of 31 kDa, was found to be homologous to a protein that has been previously described in the literature by several groups and has different names, including the 31 kDa beta-galactoside binding lectin, the IgE binding protein, and Mac-2. The multiple functions ascribed to this protein most likely relate to its ability to bind to poly-N-acetyllactosamine that is present on specific proteins. Binding of HLBP31 to laminin can be competitively inhibited by lactose, N-acetyllactosamine, or by treatment of laminin with endo-beta-galactosidase. HLBP14 has a molecular mass of 14 kDa, and was found to be homologous to the soluble 14 kDa beta-galactoside binding lectin. We have developed polyclonal rabbit antibodies to HLBP31 and to HLBP14. These antibodies also recognize the 67LR on immunoblots and in immunoprecipitation experiments, demonstrating that HLBP31, HLBP14, and the 67LR share a common epitope and possibly a similar mechanism of laminin interaction. We have also obtained specific antibodies to HLBP31 that were generated against recombinant IgE binding protein. The latter does not cross-react with the other laminin binding proteins and can be used specifically in immunohistochemistry.

Isolation of a cDNA clone of the HLBP31. Using the protein sequence information generated from HLBP31 peptides, we developed specific oligonucleotides, and reverse transcribed a specific 270 base long mRNA transcript from human A2058 melanoma cells. We then amplified this sequence by PCR, and cloned it. The cDNA clone recognizes a 1000 base long mRNA on Northern blots.

Inverse modulation of mRNA levels for the 67LR and HLBP31 in human colorectal carcinoma is correlated with metastatic potential. Using Northern and slot blot hybridization analysis, we analyzed paired tumor and normal tissues from 21 individual patients with colorectal carcinomas for their steady state levels of 67LR mRNA and HLBP31 RNA. In contrast to increased levels of 67LR mRNA in the colon tumors, steady state levels of HLBP31 mRNA were down regulated in 18 out of 21 colon carcinoma lesions compared to their corresponding normal colonic mucosa. Furthermore, both the steady state levels of HLBP31 mRNA and the ratio of HLBP31/67LR mRNA in the primary colon carcinoma lesions were significantly

($p<0.05$) lower in metastatic versus nonmetastatic colon carcinoma. Immunoblots demonstrated that HLBP31 protein is also decreased in colorectal carcinomas compared to normal colonic mucosa. The data suggest that the determination of the relative mRNA levels of HLBP31 and the 67LR may be a valuable prognostic adjunct in the evaluation of primary colon cancer lesions. Future studies will determine if the selective use of different laminin binding proteins by colonic cancer cells may play a functional role in the disease process.

In situ hybridization using specific riboprobes for the 67 kDa laminin receptor and HLBP31. We have developed a specific riboprobe for the 67LR to use in *in situ* hybridization of cytospin and tissue sections. There is an increased amount of *in situ* hybridizable mRNA for the 67LR in colorectal carcinoma cells compared to normal colonic cells. A riboprobe was recently developed for the HLBP31 and *in situ* hybridization is underway of colon carcinoma samples. The *in situ* hybridization technique will be applied to other human tumor tissues in the near future.

Publications:

Castronovo V, Taraboletti G, Sobel ME. Laminin receptor complementary DNA-deduced synthetic peptide inhibits cancer cell attachment to endothelium. Cancer Res 1991;51:5672-8.

Castronovo V, Taraboletti G, Sobel ME. Functional domains of the 67-kDa laminin receptor precursor. J Biol Chem 1991;266:20440-6.

Coopman P, Verhasselt B, Bracke M, de Bruyne G, Castronovo V, Sobel M, Foidart JM, van Roy, F, Mareel M. Arrest of MCF-7 cell migration by laminin *in vitro*: possible mechanisms. Clin Exp Metast 1991;9:469-84.

Campo E, Monteagudo C, Castronovo V, Claysmith AP, Fernandez PL, Sobel ME. Detection of laminin receptor mRNA in human cancer cell lines and colorectal tissues by *in situ* hybridization. Am J Pathol (in press)

Cioce V, Margulies IMK, Sobel ME, Castronovo V. Identification of the specific site of interaction between the 67 kilodalton metastasis-associated laminin receptor and laminin. Kidney International (in press)

Castronovo V, Luyten F, van den Brule F, Sobel ME. Identification of a 14 kD laminin binding protein (HLBP14) in human melanoma cells that is identical to the 14 kD galactoside binding lectin. Arch Biochem Biophys (in press)

Tannenbaum T, Yuspa SH, Grover A, Castronovo V, Sobel ME, Yamada Y, and de Luca LM. Extracellular matrix receptors and mouse skin carcinogenesis: altered expression linked to appearance of early markers of tumor progression. Cancer Res (in press)

Castronovo V, Campo E, van den Brule F, Claysmith AP, Cioce V, Liu F-T, Fernandez PL, Sobel ME. Inverse modulation of steady state mRNA levels of two non-integrin laminin binding proteins in human colon carcinoma. J Natl Cancer Inst (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09163-05 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Anticancer Effects *In Vitro* and *In Vivo* of a Novel Drug, CAI (NSC 609974)

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

PI:	E. Kohn	Senior Staff Fellow	MB NCI
OTHER:	W. Jacobs	Special Volunteer	LP NCI
	L. Travers	Special Volunteer	LP NCI
	K. Holmes	General Fellow	LP NCI
	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

Medicine Branch, DCT, NCI; Developmental Therapeutics Program, DCT, NCI; M.A. Sandeen, Research Technician, FCRDC, DCBDC Animal Holding Unit; C. Felder, Senior Staff Fellow, LCB, NIMH

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.4	1.6

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☐ (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 past year has seen progress in studies of CAI which include *in vitro* and *in vivo* effects, mechanism of action, and resistance to CAI. The preclinical pharmacology and CAI toxicity studies were completed with the approval of the Investigational New Drug application by the FDA, and initiation of the Phase I clinical study. Our laboratory has refined and mastered the HPLC technique for measuring CAI plasma levels and is providing patient pharmacokinetic data. The first patients studied had CAI plasma levels in the lower end of the effective *in vitro* range. Structure-activity studies have shown that dehalogenation of the hydrophobic tail completely abrogates the CAI-induced inhibition of signal transduction, proliferation, and experimental metastasis, whereas, modifications of the triazole head group has no effect. Over the past year, CAI-drug resistant A2058 human melanoma, OVCAR3 human ovarian cancer, and CHOm5 cell lines have been developed. Studies have been initiated to investigate this drug resistant phenotype. A functional endogenous muscarinic receptor has been identified on the A2058 cells. Activation of this receptor mediates reversal of the transformed phenotype, stimulates arachidonic acid release, and calcium influx. Stimulated calcium influx and internal release are inhibited by CAI treatment. Molecular and biologic techniques are in use to identify and clone this receptor. The project to identify and clone the CAI binding site has been initiated; tools for this task have been developed which include production of a ^{14}C-labelled compound and anti-CAI antibodies for expression cloning, and use of the resistant/wild type paired cell lines for protein and gene isolation. Collaborative studies have identified *in vitro* myelosuppressive effects of CAI in mouse bone marrow cultures, and mixed responses of human astrocytoma and gliomamultiforme cell lines to incubation with CAI.

Major Findings:

1. Preclinical and clinical development. During the past year, preclinical development of CAI has been completed, the IND application filed, and the clinical trial begun. Preclinical toxicity was minimal in all species tested where blood levels remained below 30 μg/ml. BID dosing in dogs was associated with several episodes of wobbly gait and 2 episodes of convulsions for which necropsy did not reveal cause. The Phase I clinical trial has been initiated with the first dose level at 100 mg/m2/d for 28 days. Escalation will proceed according to the modified Fibonacci schedule with 3 patients per level. The first patients have had no toxicity and blood levels have been in the range of 1-3 μg/ml, a level at which *in vitro* activity has been documented.

2. Development of a method for quantitation of CAI in plasma and tissues. Two independent methods have been developed for the accurate measurement of CAI. A solid phase organic extraction followed by HPLC separation using an isocratic MeOH-ammonium acetate mobile phase is used for plasma levels. A liquid phase methylene chloride-Na phosphate extraction followed by HPLC separation using an acetonitrile-tetrahydrofuran-ammonium acetate gradient mobile phase was developed for tissue and cell extractions. Both assays have excellent accuracy and reproducibility.

3. Structure/Activity Relationship. Chemical modifications of the CAI structure have been produced and tested using a screen which includes monolayer proliferation, soft agar colonization, experimental metastasis assays, arachidonic acid release, and calcium flux. Additions to the triazole head group did not prevent metastasis- and proliferation-inhibition functions of the parent compound. Substituted benzophenone structures which approximate the tail, and the tail itself, have no inhibitory activity. The dehalogenated parent compound was the only compound with altered behavior; all effects of parental CAI were completely abrogated. In all cases, the effects on proliferation and signal transduction were seen in concert, supporting the hypothesis that the mechanism of action of this drug is through inhibition of the selected signal transduction pathways.

4. Effects of CAI on hematopoiesis. In collaboration with Donna Volpe, Ph.D. of the FDA, the effects of CAI on marrow stem cell functions is under study. In vitro, CAI has marked inhibitory effects on the cloning efficiency of CFU-GM, BFU-E, and CFU-E units, with IC50s below 1 μM. Human bone marrow cultures are underway. Subsequent studies will evaluate the cloning capacity of bone marrow from CAI-treated animals.

5. Identification of endogenous muscarinic receptors (mAChR) on the A2058 human melanoma cell. A functional mAChR has been demonstrated by its ability to stimulate arachidonic acid release, calcium internal release and influx, and to inhibit colony formation in soft agar; animal studies will investigate the effect of receptor activation on tumor formation. Preliminary Northern blot analysis of A2058 RNA suggest that this receptor may be genetically related to the m1 or m2 subtypes but not the m3, m4, or m5 receptors. Further molecular and biochemical characterization of this receptor is in progress.

Publications:

Kohn EC, Liotta LA. Anti-metastastic carboxyamino imidazole (CAI). (U.S. Patent, allowed)

Felder CC, Ma AL, Liotta LA, Kohn EC. The antiproliferative and antimetastatic compound L651582, inhibits muscarinic acetylcholine receptor-stimulated calcium influx and arachidonic acid release. J Pharmacol Exp Ther 1991;257:967-71.

Kohn EC, Sandeen MA, Liotta LA. *In vivo* efficacy of a novel inhibitor of selected signal transduction pathways including calcium, arachidonate, and inositol phosphates. Cancer Res (in press)

Kohn EC. Aging issues in invasion and metastasis: Fertile ground for investigation. Cancer (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09164-05 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Role of Collagenolytic Metalloproteinases in Metastases

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

PI:	W. Stetler-Stevenson	Medical Officer	LP NCI
OTHER:	D. Kleiner	Medical Staff Fellow	LP NCI
	A. Levy	Microbiologist	LP NCI
	A. Murphy	Staff Fellow	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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.)*

In order to investigate the role of type IV collagenase in tumor invasion and metastases, we have focused on the multilevel regulation of this enzyme. These studies have shown that in contrast with other members of the collagenase enzyme family, the 72 kDa type IV collagenase mRNA levels are increased in response to TGFβ1, are unaffected by the tumor promoting phorbol esters, and show elevated levels in colorectal 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 type IV collagenase 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. Further characterization and purification of components of this activation mechanism are ongoing.

We have studied the structure of the latent enzyme TIMP-2 complex through production of enzyme deletion mutants and enzyme inhibitor cross linking studies. These studies demonstrate that the 72 kDa type IV collagenase has at least two TIMP-2 binding domains. The principal binding domain is located in the C-terminal, hemopexin-like domain of the enzyme. This binding site is available in the latent enzyme form. The second binding site is at the enzyme active site and only becomes available following organomercurial mediated enzyme activation.

Finally, antipeptide antibodies against the 92 kDa type IV collagenase, interstitial collagenase, stromelysin-1 and stromelysin-2 have been prepared and characterized.

GPO 914-918

Major Findings:

1. The 72 kDa collagenase IV enzyme is activated by organomercurial compounds *in vitro*.

2. There is specific cellular activation mechanism *in vivo*.

3. Activation results in removal of an 80 amino acid profragment peptide.

4. The 80 amino acid profragment contains a highly conserved peptide region which is responsible for maintaining the latency of the proenzyme through a sulfhydryl-metal atom interaction as determined by titration studies of the free sulfhydryls associated with the holoproenzyme and apoproenzyme preparations.

5. Synthetic peptides containing the highly conserved region from the amino terminal profragment of the 72 kDa type IV collagenase inhibit enzyme proteolytic activity against gelatin and type IV collagen *in vitro*. In addition, these peptides specifically block tumor cell invasion across reconstituted basement membranes *in vitro*.

6. The cellular activation mechanism for the 72 kDa type IV collagenase is cell surface associated; inhibited by metalloproteinase inhibitors; specific for the 72 kDa type IV collagenase enzyme; induced by specific treatments in both primary cell culture and metastatic human tumor cell lines.

7. The 72 kDa type IV collagenase is secreted as a complex with TIMP-2.

8. 72 kDa type IV collagenase has two TIMP-2 binding sites. These are located in the C-terminal hemopexin-like domain and the enzyme active site.

Publications:

Albini A, Melchiori A, Santi L, Liotta LA, Brown PD, Stetler-Stevenson WG. Tumor cell invasion inhibited by TIMP-2. J Natl Cancer Inst 1991;83:775-9.

Liotta LA, Stetler-Stevenson WG, Steeg PS. Cancer invasion and metastasis: positive and negative regulatory elements. Cancer Invest 1991;9:543-51.

Stetler-Stevenson WG, Bersch N, Golde DW. Tissue inhibitor of metalloproteinase-2 (TIMP-2) has erythroid-potentiating activity. FEBS Lett 1992;296:231-4.

Stetler-Stevenson WG, Liotta LA, Brown PD. Role of type IV collagenases in human breast cancer. In: Dickson RB and Lippman ME, eds. Breast cancer: cellular and molecular biology III. Kluwer Acad Press (in press, 1992)

Stetler-Stevenson WG, Krutzsch HC, Liotta LA. TIMP-2: Identification and characterization of a new member of the metalloproteinase inhibitor family. Matrix (Suppl 1) (in press, 1992)

Rosenberg GA, Kornfeld M, Estrada E, Kelley RO, Liotta LA, Stetler-Stevenson WG. TIMP-2 blocks proteolytic opening of blood-brain barrier by type IV collagenase. Brain Res (in press, 1992)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09179-04 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Novel Metalloproteinase Inhibitors: Role in Tumor Invasion and Metastasis

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

PI:	W. Stetler-Stevenson	Medical Officer	LP NCI
OTHER:	J. Ray	Staff Fellow	LP NCI
	A. Murphy	Staff Fellow	LP NCI
	G. D'Orazi	Visiting Fellow	LP NCI
	D. Kleiner	Medical Staff Fellow	LP NCI
	A. Levy	Microbiologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. TIMP-2 binds specifically to the latent form of the 72 kDa type IV collagenase. Recent studies have shown that all cells studied to date which secrete the 72 kDa type IV collagenase enzyme secrete this enzyme as a complex with TIMP-2. Our studies have shown that TIMP-2 transcription is regulated independently of both TIMP-1 and the 72 kDa type IV collagenase enzyme. We have also demonstrated that TIMP-2 is anti-angiogenic. The mechanism for this effect may be through inhibition of endothelial cell proliferation in addition to inhibiting endothelial cell mediated matrix proteolysis. We have shown that TIMP-2 inhibits tumor cell invasion through reconstituted basement membranes *in vitro*, and that this inhibitor demonstrates erythroid potentiating activity (EPA).

Recent studies have been directed at cloning the human TIMP-2 gene and determining its chromosomal localization. Two human TIMP-2 genomic clones of approximately 9 and 12 kb have been obtained. The gene appears to be single copy and is localized on human chromosome 17q22-25.

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.

Major Findings:

1. There is a novel 21 kDa protein which binds selectively and with 1:1 molar stoichiometry to the latent form of the human 72 kDa type IV collagenase to form a proenzyme inhibitor complex.

2. All cells which produce the 72 kDa type IV collagenase complex produce this enzyme in complexed form.

3. Studies of the transcription of TIMP-2 mRNA reveal that TIMP-2 is regulated independently from TIMP-1 and the 72 kDa type IV collagenase.

4. TIMP-2 inhibits tumor cell invasion through a reconstituted basement membrane *in vitro*.

5. TIMP-2 inhibits angiogenesis in the chick chorioallantoic membrane assay and this effect is primarily due to inhibition of endothelial cell proliferation.

6. TIMP-2 has erythroid-potentiating activity.

7. The human TIMP-2 gene is single copy and localized to chromosome 17q22-25.

8. There are two TIMP-2 binding sites on the human 72 kDa type IV collagenase.

9. The protease inhibitory domain of TIMP-2 is located in the N-terminal half of the TIMP-2 molecule.

Publications:

Stetler-Stevenson WG, Talano J, Gallagher ME, Krutzsch HC, Liotta LA. Inhibition of human type IV collagenase by a highly conserved peptide sequence derived from its prosegment. Am J Med Sci 1991;302:163-70.

Templeton N, Stetler-Stevenson WG. Identification of a basal promoter for the human M_r 72,000 type IV collagenase gene and enhanced expression in a highly metastatic cell line. Cancer Res 1991;51:6190-93.

Liotta LA, Stetler-Stevenson WG, Steeg PS. Cancer invasion and metastasis: positive and negative regulatory elements. Cancer Invest 1991;9:543-51.

Ailenberg M, Stetler-Stevenson WG, Fritz IB. Secretion of latent type IV procollagenase and active type IV collagenase by testicular cells in culture. Biochem J 1991;279:75-80.

Templeton NS, Rodgers LA, Levy AT, Ting K-L, Krutzsch HC, Liotta LA, Stetler-Stevenson WG. Cloning and characterization of a novel human cDNA that has DNA similarity to the conserved region of the collagenase gene family. Genomics 1992;12:175-6.

Corcoran ML, Stetler-Stevenson WG, Brown PD, Wahl LM. Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92-type IV collagenase/gelatinase production by human monocytes. J Biol Chem 1992;267: 515-9.

Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC. Role of the $\alpha_v\beta_3$ integrin in tumor cell invasion. Proc Natl Acad Sci USA (in press, 1992)

Kleiner DE Jr, Unsworth EJ, Krutzsch HC, Stetler-Stevenson WG. Higher-order complex formation between the 72 kDa type IV collagenase and tissue inhibitor of metalloproteinases-2 (TIMP-2). Biochemistry (in press, 1992)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09185-03 LP

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): G Proteins and Tumor Cell Motility

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

PI:	S. Aznavoorian	Biotechnology Fellow	LP NCI
OTHER:	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH: Laboratory of Pathology

SECTION: Tumor Invasion and Metastases Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	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 chemotactic response of the human melanoma cell line A2058 to type IV collagen is transduced by a pertussis toxin (PT)-sensitive G protein. However, the specific identity of the G protein involved is not known, nor are the events subsequent to G protein activation which result in pseudopodial protrusion and cellular translocation. A method has been devised for the isolation and analysis of pseudopods from A2058 cells which have been stimulated with type IV collagen. Using polycarbonate filters with extremely small pore sizes (1 μm) in a chemotaxis assay, pseudopods formed in response to attractant were isolated from the filters with SDS containing buffer, and analyzed by Western immunoblotting with a panel of antibodies specific for subclasses of Gα proteins. Results to date indicate that pseudopods formed after type IV collagen stimulation contain a PT sensitive G protein similar (or identical) to human $G\alpha_{i1}$ and $G\alpha_{i2}$ in the COOH-terminus, but different from both in another, more central region. More specific identification of the G protein in pseudopods will require 2 dimensional gel electrophoresis followed by Western blotting, and/or purification of the G protein from these extracts. In addition, progress has been made in the subcloning and sequencing of cDNAs encoding Gα subunits isolated from a λgt11 library of A2058. Partial sequence information on one insert has confirmed it to be the human $G\alpha_{i2}$-encoding cDNA. Many other $G\alpha_{i1}$- and $G\alpha_{i2}$-like subclones are available for sequence analysis, or possibly for probing with specific antibody in the λgt11 expression vector, to search for the relevant one in type IV collagen-mediated chemotaxis.

Major Findings:

Chemotaxis of tumor cells involves many complex and incompletely understood steps, including binding of attractant to its specific receptor, transduction of a signal across the membrane, activation of internal effectors, pseudopodial protrusion, and ultimately, cellular translocation. It has been previously established that the chemotactic response of A2058 cells to type IV collagen is transduced by a pertussis-toxin (PT)-sensitive G protein; however, its specific identity is not known. Presumably, activation of this G protein leads to activation of internal effector enzymes and/or ion channels which cause disassembly, then reassembly of actin to form pseudopods in the direction of attractant. It is not completely understood how the binding of attractant to receptor leads to such rearrangements of the cytoskeleton. Previous work with neutrophils has indicated that all the necessary components for motility (receptors, transducers, effectors) are located in pseudopods. Therefore, a method was devised for isolation and analysis of pseudopods from cells stimulated with type IV collagen. In a chemotaxis assay using filters of only 1 μ pore size (typical pore size for a migration assay is 8 μ), we found that we could isolate pseudopods from the remainder of the cell body. The pseudopods are extracted from the filter with SDS-containing buffer, and analyzed by Western immunoblotting. A panel of antibodies specific for subclasses of G protein α subunits has been used in an attempt to identify the G protein in type IV collagen-stimulated pseudopods. An antiserum to the COOH-terminal decapeptide common to $G\alpha_{i1}$ and $G\alpha_{i2}$ recognized a protein of ~40 kd in pseudopod extract, as well as in a whole cell extract. However, an anti-peptide antibody specific for $G\alpha_o$ recognized the appropriately sized protein in whole cell extract, but not in pseudopods, leading to the speculation that $G\alpha_o$ is not involved in this pathway for motility, and is therefore "sequestered out" of pseudopods. Interestingly, an anti-peptide antibody specific for a central region of human $G\alpha_{i2}$ (amino acids 113-126) recognized its antigen only in whole cell extract, and not in pseudopods; apparently, pseudopods contain a PT sensitive G protein which has a COOH-terminus common to that of human $G\alpha_{i2}$, but which differs at least to some degree in the region of 113-126 (or else is covalently modified in this region, for example by phosphorylation). To date, an antibody to a peptide unique to $G\alpha_{i1}$ has not recognized its antigen in either whole cells or pseudopods; however, higher concentrations of this antibody, and possibly antibodies to different regions of $G\alpha_{i1}$, must be tested before we can rule out the presence of $G\alpha_{i1}$ in pseudopods. Specific identification of the G protein in pseudopods will probably require analysis by 2-dimensional gel electrophoresis, and/or purification of the protein from these extracts.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09352-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

AAMP-1, Activation Associated Motilty Protein-1

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

PI:	M. Beckner	Biotechnology Fellow	LP NCI
OTHER:	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

A cDNA clone encoding a human melanoma cell protein, AAMP-1, has been isolated using a monoclonal antibody, 1AA3, which was selected from earlier screening studies involving inhibition of tumor cell motility. The mAB, 1AA3, reacts strongly with a 95 kD protein and moderately with a 44 kD protein on immunoblots of A2058 whole cell lysates. It is possible that the 44 kD protein is a subunit of the larger protein. AAMP-1, the cDNA clone that we have sequenced, predicts a novel protein that is 44 kD. It has a potential transmembrane domain and exhibits significant homology (ALIGN Scores greater than 3.0, S.D. Protein Identification Resource program) to numerous immunolgobulin superfamily members (Ig kappa, Ig lambda, CD28, CD4, CD7, CD2, poly Ig receptor, NCAM, T cell receptor alpha, MAG, vaccinia virus, HT7, and link protein). Its single 1.6 Kb mRNA transcript is increased in activated human T cells. It is expressed in many human cell types, including metastatic melanoma cells, metastatic breast carcinoma, macrophages, fibroblasts, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Several AAMP-1 regions have been found to have strong local homology with HIV-1 proteins including a region important in CD4 binding. These regions are "immunoglobulin-like" and these regions in AAMP-1 have been selected for further study with antipeptide antibodies. Currently, the AAMP-1 gene is being subcloned into a suitable vector for recombinant protein production of its 44 kD predicted product.

The 95 kD protein mentioned earlier has been partially purified.

A gene, approximately 1907 bp related to the AAMP-1 gene, has been isolated for further study.

Future Plans:

1. Purification of the 44 kD AAMP-1 recombinant protein for structure-function analysis, for production of domain specific antibodies to AAMP-1, and for use in motility assays of cancer cells, activation assays of various cell types, and cell binding assays.

2. Determine the pattern of AAMP-1 mRNA expression in activation assays of cells of low and high metastatic potential.

3. Develop antipeptide antibodies to AAMP-1, including the site with homology to the CD4 binding site of HIV-1, for functional studies of AAMP-1.

4. Complete purification of the 95 kD protein initially identified by 1AA3.

5. Sequence the 1907 bp gene that is related to AAMP-1.

6. Determine the genomic sequence for AAMP-1.

7. Continue studies of AAMP-1's tissue distribution and cell localization with specific antibodies when they are available.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09353-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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	Senior Investigator	LP NCI
OTHER:	V. Castronovo	Visiting Scientist	LP NCI
	F. van den Brule	Visiting Fellow	LP NCI
	C. Oktrakji	Special Volunteer	LP NCI
	J. Price	Special Volunteer	LP NCI

COOPERATING UNITS *(if any)*

Dr. A. Berchuck, Duke University, North Carolina; Dr. F.-T. Liu, Scripps Clinic, California; Dr. T. Broker, University of Rochester School of Medicine, New York

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

One of the major challenges of cancer research is to define new methods for the detection of cancer lesions and to predict the aggressiveness and the metastatic potential of an individual patient's tumor. Such methods could help in the assessment of the best therapeutic strategy for a given patient. We have initiated a series of survey studies of breast, ovarian, and cervical 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 the 67 kDa high affinity laminin receptor (67LR), a 31 kDa laminin binding protein with lectin binding properties (HLBP31), as well as other genes that are thought to play a pathophysiological role in tumor cell invasion, including collagenases. Freshly frozen tumor samples and matched normal tissues 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 fixed and frozen tissues. Results will be correlated with the survival of cancer patients to establish the prognostic value of the systematic detection of these genes in gynecological tumors. As an adjunct to these survey studies, *in vitro* experiments are being conjucted to determine the specific effect of steroid hormones on human breast cancer cells. Preliminary evidence suggests that different types of cancer cells express specific laminin binding proteins, and that in breast cancers, 67LR and HLBP31 are inversely modulated. In cervical lesions, adenomatous cancers express more 67LR; however, squamous carcinomas do not differentially express this gene. In *in vitro* studies of steroid receptor-positive human breast cancer cell lines, 67LR appears to be upregulated by both estrogen and progesterone, while HLBP31 is responsive only to progesterone. These data demonstrate that estradiol and progestins differentially modulate the expression of genes involved in critical steps of cancer invasion and metastasis in human breast cancer cells.

Major Findings:

Modulation of gene expression in human breast cancer cell lines by steroid hormones. We have previously shown that the expression of the 67LR is increased in the steroid receptor-negative human breast cancer cell line MDA-MB231 in contrast to the steroid receptor-positive line T47D. Expression in the latter cell line can be increased by growth in the presence of estrogen and progesterone. We have expanded the initial study by preparing new samples from MDA-MB231 and T47D cells, as well as the estrogen-responsive human breast cancer cell line MCF7. Protein and RNA expression of the 67LR, HLBP31, type IV collagenase (MMP-2) and its inhibitor TIMP-2 were studied in all cell lines grown in the absence and presence of steroid hormones. As expected, 67LR expression was increased by estrogen and/or progesterone in the T47D cells, and by estrogen in the MCF7 cells. In contrast, HLBP31 expression was not affected by estrogen, but was increased by progesterone. MMP-2 levels were not altered by steroid treatment; however, progesterone caused a down-regulation of TIMP-2 expression. This would cause a net increase in collagenolytic activity produced by the breast cancer cells. The data demonstrate that the 67LR, HLBP31, MMP-2, and TIMP-2 genes have different regulatory elements that are differentially affected by specific steroids, and suggest that estrogens and progestins may modulate the expression of genes involved in critical steps of cancer invasion and metastasis in steroid receptor positive human breast cancer cells in patients.

Expression of laminin binding proteins in human ovarian cancer samples. Twenty ovarian tumors and a normal ovary were obtained from Duke University. Total protein extracts and intact total cellular RNA were prepared from all samples. Expression of the 67LR was increased significantly in the ovarian cancer samples; however, HLBP31 expression was more variable.

Inverse modulation of HLBP31 and 67LR expression in human breast cancers. Using specific antibodies to HLBP31 and to 67LR, a series of fixed human breast cancer specimens was evaluated immunohistochemically. Expression of 67LR antigen was high in the cancer specimens; however, HLBP31 antigen was dramatically decreased in the cancer cells compared to the adjacent normal breast tissue. These data are important in light of recent reports from our laboratory of a similar inverse modulation between the two laminin binding proteins in human colon cancer. Thus, cancer cells may express different laminin receptors on their surface compared to normal cells, possibly affecting their ability to detach and invade the adjacent stroma.

Differential expression of 67LR in human papillomavirus-associated cervical neoplasms. Histopathological sections of a large number of epithelial lesions of the genital tract associated with human papillomaviruses were assessed for 67LR expression by *in situ* hybridization. In squamous tissues, 67LR was expressed primarily in the less differentiated cells and in a spectrum of neoplasms; however, there was no net induction of mRNA per cell in intraepithelial or invasive squamous neoplasms relative to normal tissue. In contrast, 67LR mRNA was not expressed at a detectable level in normal glands of the cervix, but was dramatically induced in abnormal, human papillomavirus-positive glands. Increased 67LR expression appeared to correlate with proliferative rather than with the invasive properties of these cells.

Publications:

Demeter LM, Stoler MH, Sobel ME, Broker TR, Chow LT. Expression of high affinity-laminin receptor mRNA correlates with cell proliferation rather than invasion in human papillomavirus-associated cervical neoplasms. Cancer Res 1992;52:1561-7.

Noel MC, Sobel M, Foidart JM. Expression of laminin by human fibroblasts, HT1080 fibrosarcoma cells and MCF-7 breast adenocarcinoma cells: lack of regulation by the cell density and extracellular matrix. Cell Biol Int Rep 1991;15:499-509.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09367-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Identification of Cancer-Specific Homeobox Genes

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

PI:	V. Castronovo	Visiting Scientist	LP NCI
OTHER:	M. Sobel	Senior Investigator	LP NCI
	M. Kusaka	Guest Researcher	LP NCI
	A. Chariot	Special Volunteer (MOI)	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	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.)*

Homeobox genes, encoding transcriptional regulators, act in complex regulatory cascades to control the coordinated expression of genes involved in specific developmental processes. Originally identified and studied in *Drosophila*, homeobox genes have now been isolated from a variety of vertebrate species, including human. We have asked the question whether specific homeobox genes may control the coordinated expression of genes involved in human cellular transformation and in tumor invasion and metastasis. We have targeted human breast cancer as an initial model system. We have taken advantage of the fact that all known homeobox genes in all species share a common 180 bp segment of DNA that encodes a highly conserved 60 amino acid domain responsible for binding to DNA. Using degenerate primers within this common DNA segment and polymerase chain reaction technology, we amplified homeobox gene segments from RNA that had been extracted from a variety of human breast cancer cell lines. We identified unique homeobox sequences that are not present in the Genebank. Current efforts are directed toward isolating full length cDNA clones of these unique genes to determine their complete sequences and regulatory functions in breast cancer.

GPO 914-918

Major Findings:

Identification and cloning of a homeobox DNA binding domain in human breast cancer cell lines. All known homeobox genes share a common 180 bp segment of DNA that encodes a highly conserved 60 amino acid domain responsible for binding to DNA. To test whether human breast cancer cells express homeobox mRNA(s), we used degenerate primers derived from the homologous homeobox DNA binding domain and reverse transcriptase-polymerase chain reaction technology to test RNA that was isolated from human breast cancer tissue as well as RNA isolated from human breast cancer cell lines. We amplified 130 bp DNA fragments from all the human breast cancer RNA preparations. By adding specific restriction enzyme recognition sequences to the ends of the 130 bp fragment(s), we were able to generate a specific human breast cancer homeobox DNA binding domain cDNA library in the prokaryotic vector pBluescript KS. Preliminary sequence analysis of 20 independent clones revealed that there were at least 6 different 130 bp fragments, all of which showed similarity to known homeobox genes. However, 5 of the 6 clones had sequences sufficiently dissimilar from known genes to lead to the conclusion that they represent newly discovered homeobox genes.

Future directions. To determine the biological relevance of these preliminary findings, we are pursuing two experimental lines. (1) First, we are attempting to isolate full length cDNA clones representative of all the expressed homeobox genes in human breast cancer. To achieve this, we have constructed a cDNA library from RNA isolated from the well known human breast cancer cell line MCF7. We are screening the library with a mixture of 5 different 130 bp homeobox DNA binding probes. To date, we have purified 36 cDNA clones from the MCF7 library. We will first verify that they contain homeobox cDNA fragments and then sequence these clones to further assess their identity and possible uniqueness. (2) Second, we will determine the expression of specific homeobox mRNA species in human breast cancers by measuring homeobox mRNA content. This will require a very sensitive and specific assay since homeobox mRNAs are very rare and have a high degree of sequence similarity. Thus, we are developing a homeobox RNAse protection assay to quantitatively measure specific homeobox sequences in human breast cancers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09368-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Molecular Pathology Resource Center

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

PI:	M. Sobel	Senior Investigator	LP NCI
OTHER:	L. Wrathall	Biologist	LP NCI
	J. Price	Special Volunteer	LP NCI
	C. Otrakji	Special Volunteer	LP NCI
	M. Kusaka	Guest Researcher	LP NCI
	P. Fernandez	Visiting Fellow	LP NCI
	A. Chariot	Special Volunteer (MOI)	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.4	0.6

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 is being developed to coordinate the training of residents, intramural staff, and extramural scientists on the application of molecular pathology techniques to the study of cancer biology. The center will provide a resource with tissue specimens for the analysis of gene expression in neoplasia. In addition, bench and didactic training in molecular techniques will be provided. During the first 6 months of developing the resource center, we have provided training to two extramural scientists, a high school teacher, a pharmacist and a Visiting Fellow.

Major Accomplishments:

During the planning stages of the resource center, we have begun to provide training to extramural and intramural scientists. Training includes bench experience with techniques including Northern blot, Southern blot, immunoblot, RNA and DNA extraction, in situ hybridization, probe labeling, and tissue handling. In addition, participants participate in a weekly molecular pathology journal club as well as weekly data review sessions. Didactic lectures on selected topics are also provided several times a week. We have provided training to a high school biology teacher, a pharmacist, visiting scientists from Japan and Miami, as well as a Visiting Fellow in the Surgical Pathology Section of the Laboratory of Pathology and a laboratory chief from the NIH Clinical Center. Training is provided for short (three months) as well as long time periods (one year) depending on the needs of the participant. In addition, equipment for retrieval and storage of tissues for analysis are being acquired for the future expansion of this program.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09369-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Regulation of Ribonucleotide Reductase in Human Cancer Cells

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

PI:	M. Sobel	Senior Investigator	LP NCI
OTHER:	K. Barker	Special Volunteer	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	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.)*

Ribonucleotide reductase catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, a rate limiting step of DNA synthesis. Mammalian ribonucleotide reductase is composed of two subunits: M1, which is constitutively expressed throughout the cell cycle, and M2, whose expression is S-phase dependent. In the course of studying differentially expressed genes in human cancer, we isolated full length clones of the M2 subunit of ribonucleotide reductase from a human melanoma A2058 cell cDNA library. Northern blot analysis demonstrated that there are two major mRNA transcripts of human M2, with sizes of 1.6 and 3.4 kb. Sequence data showed that the two transcripts differ in the 3′ untranslated region due to alternate use of polyadenylation sites. The cDNA clones have an open reading frame encoding 389 amino acids, one less codon than found in the murine protein. The cDNA-predicted human protein sequence is 91% similar to that of the mouse; however, the 5′ and 3′ untranslated mRNA sequences are totally dissimilar to the murine analog. Northern and slot blot analyses performed on RNAs isolated from Dukes' C and D human colorectal carcinomas showed a 1.8-fold increase in M2 mRNA expression in tumors compared to paired normal adjacent colonic tissue. The data are consistent with the hypothesis that M2 mRNA activity is increased to provide rate limiting precursors for DNA synthesis in proliferating colonic cancer cells.

Major Findings:

Sequence determination of human M2 mRNA. Using a 356 bp region of a laminin receptor pseudogene that recognized two major mRNA transcripts of human RNA on Northern blot, we screened a human melanoma cell cDNA library for full length clones. The probe contained a unique sequence that was not known in the Genebank and that did not contain an open reading frame. Among the cDNA clones selected by screening the human melanoma library, we isolated three clones that contained upstream sequences with an open reading frame. By comparison to the Genebank, we identified the clones as encoding the human analog of the murine M2 (catalytic) subunit of ribonucleotide reductase. By comparison to the murine sequence, the coding sequence of the human M2 was 91% similar. Notably, the human protein has one less amino acid than the mouse polypeptide. The 5′ and 3′ untranslated regions of the human M2 mRNA have no similarity to the mouse RNA. The 356 bp probe used to screen for the clones was found to represent the beginning of the 3′ untranslated region of the human gene.

Differential expression of human M2 mRNA in human cancers. Northern blot analysis of RNA from diploid human cells as well as from human cancer cells identified two major mRNA transcripts, of 1.6 and 3.4 kb. The levels of expression of these transcripts were examined in normal and cancer tissue from 12 patients with colon adenocarcinoma. There were increased transcript levels, varying from two- to six-fold, in the cancer tissues compared to the paired adjacent normal mucosa. The increased expression is not specific for colon cancers. In surveying ovarian cancers, 4 out of 5 ovarian adenocarcinomas had increased levels of M2 mRNA compared to normal ovarian tissue. These data suggest that proliferating cells typical of human cancers have increased levels of M2 mRNA, thus facilitating DNA synthesis.

Future directions. To determine the elements that control human M2 gene expression, we will use the current cDNA clones to isolate genomic clones of human M2. We will study the promoter and other regulatory regions of the human gene, and isolate any specific transcription factors that work on this S-phase dependent gene. Furthermore, recombinant methods will be used to explore possible diagnostic and therapeutic implications.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00550-12 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

PI:	E. Jaffe	Chief, Hematopathology Section	LP NCI
OTHER:	L. Medeiros	Senior Staff Fellow	LP NCI
	D. Longo	Senior Investigator	BRMP NCI
	M. Raffeld	Senior Staff Fellow	LP NCI
	M. Stetler-Stevenson	Senior Staff Fellow	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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:

A series of studies were completed analyzing the interrelationship between Hodgkin's disease and the non-Hodgkin's lymphomas. While Hodgkin's disease and the non-Hodgkin's lymphomas have long been regarded as distinct disease entities, recent observations suggest a closer association. The analysis of cases in which these diagnoses are made in the same anatomic site (composite lymphomas) or in separate sites (simultaneous or sequential HD and NHL) indicates that this phenomenon occurs more frequently than would be expected by chance alone. The vast majority of the NHL associated with HD are of B-cell origin, most commonly follicular lymphomas. An association between HD and B-cell CLL is also observed. These findings suggest that, at least in some cases, HD may be clonally related to an underlying B-cell malignancy, in that the Reed-Sternberg cell may be an altered B lymphocyte. A process that may have a different pathogenesis is the late occurrence of aggressive B-cell lymphomas in patients successfully treated for HD. This phenonemon most likely relates to an underlying and persistent immunodeficiency in these patients, and does not necessarily suggest a clonal relationship between the two tumors.

A study was completed which analyzed the role of CD5 expression in B-cell small lymphocytic malignancies, and its correlation with clinical presentation and sites of disease. Three subtypes of low-grade non-Hodgkin's lymphoma were analyzed: small lymphocytic (23 cases); small lymphocytic with plasmacytoid differentiation (10 cases); and lymphocytic lymphoma of intermediate differentiation (29 cases). Lack of CD5 expression was significantly associated with extranodal presentation among the overall study group ($p < 0.001$), as well as for those with small lymphocytic lymphoma and IDL, but not for those presenting with plasmacytoid tumors ($p < 0.21$). Retrospective review of the 11 extranodal cases that were CD5 negative demonstrated common histologic features characteristic of lymphomas of mucosal associated lymphoid tissue. This study supports the concept that at least two antigenically distinct B-cell subpopulations may be involved in the pathogenesis of low-grade lymphocytic malignancies.

A unique syndrome was described of T-cell lymphoblastic lymphoma with eosinophilia associated with subsequent myeloid malignancy. Two of three patients died of acute myeloid leukemia within 18 months of the diagnosis of lymphoblastic lymphoma. The third patient relapsed with a lymphoma that had histologic and immunophenotypic features of both T-cell lymphoblastic lymphoma and granulocytic sarcoma, and also developed a poorly defined myeloproliferative disorder.

Publications:

Jaffe ES, Travis WD. Lymphomatoid granulomatosis (angiocentric immunoproliferative lesions) and lymphoproliferative disorders of the lung. In Lynch JP, DeRemee R, eds. Immunologically mediated pulmonary disease. Philadelphia: JB Lippincott Co, 1991;274-301.

Devaney K, Jaffe ES. The surgical pathology of gastrointestinal Hodgkin's disease. Am J Clin Pathol 1991;95:794-801.

Gonzalez CL, Medeiros LJ, Jaffe ES. Composite lymphoma: A clinicopathologic analysis of nine patients with Hodgkin's disease and B-cell Hodgkin's lymphoma. Am J Clin Pathol 1991;96:81-9.

Travis LB, Gonzalez CL, Hankey BF, Jaffe ES. Hodgkin's disease following non-Hodgkin's lymphoma. Cancer 1992;69:2337-42.

Jaffe ES, Raffeld M. Lymphocyte markers in solid tissue. In: Rose NR, de Macario EC, Fahey JL, Friedman H, Penn GM , eds. Manual of clinical laboratory immunology, 4th ed. Washington, D.C.: American Society for Microbiology, 1992;288-97.

Sundeen JT, Longo DT, Jaffe ES. CD5 expression in B-cell small lymphocytic malignancies: Correlations with clinical presentation and sites of disease. Am J Surg Pathol 1992;16:130-7.

Sander CA, Jaffe ES, Gebhardt FC, Yano T, Medeiros LJ. Mediastinal lymphoblastic lymphoma with an immature B-cell immunophenotype. Am J Surg Pathol 1992;16:300-5.

Taubenberger JK, Jaffe ES, Medeiros LJ. Thymoma with abundant L26-positive 'asteroid' cells. A case report with an analysis of normal and thymoma specimens. Arch Pathol Lab Med 1991;115:1254-7.

Mandava SK, Medeiros LJ, Naylor P, Fowler D, Jaffe ES, Stetler-Stevenson M. Locally invasive (malignant) thymoma associated with T-cell lymphocytosis: A case report with immunophenotypic analysis. Arch Pathol Lab Med (in press)

Zarate-Osorno A, Medeiros LJ, Jaffe ES. Hodgkin's disease coexistent with plasma cell dyscrasia. Arch Pathol Lab Med (in press)

Banks PM, Chan J, Cleary ML, Delsol G, DeWolf-Peeters C, Gatter K, Grogan TM, Harris NL, Isaacson PG, Jaffe ES, Mason D, Pileri S, Ralfkiaer E, Stein H, Warnke RA. Mantle cell lymphoma: A proposal for unification of morphologic, immunologic and molecular data. Am J Surg Pathol (in press)

Abruzzo LV, Jaffe ES, Cotelingam JD, Whang-Peng J, Del Ducs V, Jr, Medeiros LJ. T-cell lymphoblastic lymphoma with eosinophilia associated with subsequent myeloid malignancy. Am J Surg Pathol 1992;16:236-45.

Jaffe ES, Zarate-Osorno A, Medeiros LJ. The interrelationship of Hodgkin's disease and non-Hodgkin's lymphomas--lessons learned from composite and sequential malignancies. Semin Diag Pathol (in press)

Jaffe ES, Raffeld M, Medeiros LJ, Stetler-Stevenson M. An overview of the classification of non-Hodgkin's lymphomas: an integration of morphologic and phenotypic concepts. Cancer Res (suppl) (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 00855-10 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
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
	J. Medeiros	Senior Staff Fellow	LP NCI
	L. Roman	Medical Staff Fellow	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.20	0.15	0.05

CHECK APPROPRIATE BOX(ES)

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

A

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

Pathologic material from patients identified to be seropositive for HTLV-I is reviewed and correlated with clinical and epidemiologic features of disease. Material is derived from patients in the United States as well as other parts of the world. Where possible, immunologic phenotyping of the lymphomas is performed and tumor DNA is directly analyzed for viral genome.

For cases in which fresh material is not available, DNA will be extracted from paraffin sections and examined for HTLV-I sequences using the PCR amplification technique. This information will be correlated with serologic, clinical and pathologic data to determine the validity of the PCR technique in establishing the diagnosis of adult T-cell lymphoma/leukemia (ATL).

In selected populations where HTLV-I is endemic, such as Jamaica or Trinidad, prospective studies of all newly diagnosed lymphoma patients are conducted. Such studies are useful in identifying the clinicopathologic spectrum of HTLV-I associated diseases. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are included to discern factors which may have an impact on the incidence of HTLV-I and HTLV-I associated diseases.

Other diseases are being investigated with respect to a possible viral association: angiocentric immunoproliferative disorders (lymphomatoid granulomatosis), sinus histiocytosis with massive lymphadenopathy, systemic Castleman's and Kikuchi's disease. Viruses under investigation include EBV, HHV-6, HTLV-I, and HTLV-II.

Major Findings:

Several studies were completed this year concerning the role of EBV in lymphoproliferative diseases. A role for EBV was confirmed in the angiocentric immunoproliferative lesions. In a molecular analysis of AIL, although these lesions do not show clonal rearrangements of the antigen receptor genes, EBV does appear to be present in some cases in a clonal form. Using Southern blot analysis, 2 of 8 cases of AIL contained clonal EBV when analyzed by probes to the terminal repeat region. Both of these cases were grade 3 lesions. A subsequent study employing *in situ* hybridization for EBV in paraffin sections demonstrated very strong positivity for EBV in virtually all of the neoplastic cells in 5 of 5 grade 3 lesions. Only 3 of 9 grade 1 or grade 2 lesions showed scattered EBV positive cells by *in situ* hybridization. These studies suggest that EBV may play a role in the histologic progression of AIL.

A series of 23 cases of angioimmunoblastic lymphadenopathy (AILD) were studied for Epstein-Barr virus using both the polymerase chain reaction technique (PCR) and *in situ* hybridization. Eighty percent of the cases were positive for EBV by PCR and positive cells could be demonstrated by *in situ* hybridization in 96% of the cases. In 18% of the biopsies (5 cases), there was very strong positivity for EBV by *in situ* hybridization with greater than 100 positive cells per medium powered field. By double-staining techniques, the vast majority of the EBV positive cells also expressed the CD20 antigen, a B-cell associated antigen, as determined by staining with L26. In addition, a case of B-cell lymphoma occurring in a patient with AILD was found to be strongly positive for EBV, with at least two different B-cell clones identified by terminal repeat region analysis. This study suggested that EBV-expanded clones may occur in AILD, possibly secondary to the immunodeficiency associated with this disease. This clonal expansion may lead, in rare cases, to the development of B-cell lymphoma, analogous to the development of B-cell lymphomas occurring in other immunodeficient states. EBV was not identified in the T cells, which form the predominant component of the lesion and are often clonal by T cell gene rearrangement analysis.

An *in situ* hybridization study for herpesvirus-6 (HHV-6) was conducted in a series of lymphoma specimens from Chinese patients. This study demonstrated the feasibility of staining cells containing HHV-6 genomes in paraffin sections, but demonstrated a low prevalence of positive cells in these cases. Thus, HHV-6 is unlikely to play a role in the pathogenesis of malignant lymphoma in this population. A very high prevalence of HHV-6 was identified in tissues involved by sinus histiocytosis with massive lymphadenopathy. The positive cells appeared to be predominantly the atypical histiocytes characteristic of this disease. Therefore, HHV-6 may play a role in the pathogenesis of Rosai-Dorfman disease.

Publications:

Medeiros LJ, Peiper SC, Elwood L, Yano T, Raffeld M, Jaffe ES. Angiocentric immunoproliferative lesions: A molecular analysis of 8 cases. Hum Pathol 1991;22:1150-7.

Medeiros LJ, Jaffe ES, Chen Y-Y, Weiss LM. Localization of epstein-Barr viral genomes in angiocentric immunoproliferative lesions. Am J Surg Pathol 1992;16:439-47.

Weiss LM, Jaffe ES, Liu X-F, Chen Y-Y, Shibata D, Medeiros LJ. Detection and localization of Epstein-Barr viral genomes in angioimmunoblastic lymphadenopathy and angioimmunoblastic lymphadenopathy-like lymphoma. Blood 1992;79:1789-95.

Yin SY, Ming HA, Jahan N, Manak M, Jaffe ES, Levine PH. *In situ* hybridization detection of human herpesvirus-6 in biopsies from Chinese patients with non-Hodgkin's lymphoma. Arch Pathol Lab Med (in press)

Levine PH, Jahan N, Murari P, Manak M, Jaffe ES. Detection of human herpesvirus-6 in tissues involved by sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease). J Infect Dis (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09181-04 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of Oncogenes in Lymphoproliferative Disorders

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

PI:	M. Stetler-Stevenson	Senior Staff Fellow	LP NCI
OTHER:	J. Wang	General Fellow	LP NCI

COOPERATING UNITS *(if any)*
Medicine Branch, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

We have found a correlation between high expression of bcl-2 and sustained complete response to therapy in a series of follicular lymphoma patients. In the same series, high expression of c-myc was associated with greater diffuse morphology and a trend towards better response to therapy.

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

Major Findings:

1. The level of expression of bcl-2 correlates with response to therapy.

2. A trend between high levels of expression of c-myc and complete response to therapy has been observed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09182-04 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

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	Senior Staff Fellow	LP NCI
OTHER:	T. Yano	Visiting Fellow	LP NCI
	C. Sander	Guest Researcher	LP NCI
	D. Kingma	Clinical Associate	LP NCI
	T. Greiner	Clinical Associate	LP NCI
	A. Karameris	Special Volunteer	LP NCI
	E. Jaffe	Chief, Hematopathology Section	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	2.4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A,B

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

The overall goal of this project is to generate molecular profiles of the lymphoproliferative disorders and to assess the usefulness of these profiles in providing clinically relevant information that may have both diagnostic and prognostic value.

Molecular profiles are generated using genetic loci that have been associated with particular lymphoproliferative disorders such as bcl-1, bcl-2 and c-myc as well as other loci whose influence in these disorders are not so well understood. The data generated for each locus is correlated with clinical, immunologic, and pathologic data. Using this combined approach, we hope to increase our diagnostic and prognostic precision in the classification of lymphopoietic disorders.

The molecular profiles also generate important biologic information with regard to the particular genes under study. Structural analysis of an abnormal gene or gene product allows us to acquire information concerning the functioning of that gene in the lymphoma and its effect on the biologic behavior of the lymphoma cell.

Sensitive molecular techniques are being developed in order to improve our ability to diagnose minimal disease, monitor the effect of therapy, and predict recurrences. Techniques designed to make better use of routine pathologic materials, particularly fixed paraffin embedded tissues, are being developed. Non-radioactive approaches are being explored so that molecular diagnostics may be performed in routine clinical laboratories as well as in academic centers.

Major Findings:

We have determined the frequency with which each of the breakpoint regions of bcl-2 are involved in translocation and are continuing to investigate whether the specific breakpoints might influence the clinical behavior of lymphoma. Follicular lymphomas that are negative for bcl-2 rearrangement are being studied for expression of bcl-2 as part of an effort to understand the differences between the bcl-2 rearranged and non-rearranged cases.

To adapt PCR technology to the diagnosis of t(14:18) translocated lymphomas, we have developed sets of oligonucleotide primers specific for each of four reported breakpoint clusters so that the majority of t(14:18) translocated lymphomas can be identified. We are continuing to study the feasibility of using PCR to follow response to therapy and predict relapse using peripheral blood samples and other tissue samples. This project has now been incorporated into a new NIH Clinical Oncology protocol to begin in the summer of 1992. We are continuing retrospective studies using paraffin embedded tissues.

We have developed technologies to extract PCR quality DNA from paraffin embedded tissues fixed in B5, an important breakthrough for our retrospective studies.

We have also extended our ability to study clonal populations by adapting and combining PCR analysis and denaturing gradient gel electrophoresis for the analysis of T cell receptor gamma gene rearrangements. We are initiating parallel studies for the immunoglobulin heavy chain gene.

We previously reported that the bcl-1 major breakpoint region is associated with mantle cell lymphoma. We have now completed a larger study that examines several additional minor breakpoint regions and we have also studied the expression of the bcl-1 related gene bcl-1/PRAD1. These studies have shown that bcl-1 rearrangement and bcl-1/PRAD1 expression are specific to the mantle cell lymphomas.

Previously we performed a molecular study of the small non-cleaved lymphomas and showed that molecular differences exist between the Burkitt's subgroup and the non-Burkitt's subgroup. In the course of these studies, we identified an aggressive subgroup of lymphomas associated with particular abnormalities of the MYC gene. We are continuing to explore the nature of these abnormalities and their effect on the biologic function of the gene.

We are continuing with studies into possible viral involvement in several lymphoproliferative diseases. These include the involvement of HTLV-like viruses in T cell diseases and the involvement of herpes viruses in cutaneous lymphoproliferative disorders, angiocentric lymphomas, AILD, and Kikuchi's disease. (Cross reference with annual report of ES Jaffe).

Publications:

Medeiros LJ, Van Krieken JH, Jaffe ES, Raffeld M. Association of bcl-1 rearrangements with lymphocytic lymphoma of intermediate differentiation pathogenesis. Blood 1990;76:2086-90.

Raffeld M, Jaffe ES. bcl-1, t(11;14) and mantle cell derived lymphomas. Blood 1991;78:259-63.

Raffeld M, Sander CA, Yano T, Jaffe ES. Mantle cell lymphoma: an update. Lymphoma & Leukemia (in press)

Yano T, Van Krieken JHJM, Magrath IT, Longo DL, Jaffe ES, Raffeld M. Histogenetic correlations between subcategories of small non-cleaved cell lymphomas. Blood 1992;79:1282-90.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09191-03 LP

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Biology of Disease Progression in Lymphoproliferative Diseases

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

PI:	M. Raffeld	Senior Staff Fellow	LP NCI
OTHER:	T. Yano	Visiting Fellow	LP NCI
	C. Sander	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.6	

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 group characterized by a single molecular lesion (bcl-2 translocation and deregulation), because over 70% of these low grade lymphomas will evolve into a histologically distinct high grade lymphoma, and because it is the most common low grade lymphoma in the United States.

We have accumulated a large series of progressed follicular lymphomas from patients that have had multiple biopsies over the course of their disease. The matched biopsies from individual patients are studied for a variety of phenotypic and genotypic charcteristics. A major focus is to identify acquired alterations within genes that have been previously implicated in growth and cell cycle control (e.g.: MYC, P53, Rb and PCNA). Changes in expression levels are also studied.

In order to identify additional genetic loci that may play a significant role in lymphoma progression, we are proceeding with the development of subtraction c-DNA libraries specific to the progressed follicular lymphoma cells from individual patients. Using this approach, we hope to identify additional critical genes that may be important to the progressed phenotype.

Major Findings:

Previously we found that the bcl-2 gene itself was unaffected by progression and that other genes must be contributing to the dramatic changes in cellular morphology and biologic behavior that occur following transformation. In the past year, we have completed initial studies on the involvement of c-myc and have found acquired, progression related structural changes in this gene in approximately 10% of progressed lymphomas. The precise molecular change and its biologic significance is being pursued.

We have recently completed another study on the involvement of the bcl-3 (17q22) gene (another anecdotally reported progression related locus) and again have found abnormalities of this locus in 10-15% of the progressed lymphomas. In contrast to the situation for c-myc, these changes are not temporally associated with the progression event, and we have concluded that alterations of this gene do not directly result in progression but may predispose to progression.

We are also nearing completion of a study of the role of the P53 gene in progression using SSCP and sequencing technology. It appears that this gene (which has been associated with progression in non-lymphoma systems) is not an important progression related gene in the follicular lymphomas.

Other genes and cofactors that do not appear to have a role in progression include the CLL progression related gene bcl-3 (19q13) and EBV.

Additional genes currently under investigation include c-rel, Rb, PCNA and the ras family of genes. Preliminary studies suggest dramatic changes occurring in the percent of cells expressing PCNA following progression and we are currently investigating whether we can use PCNA expression as a prognostic marker to predict progression.

We are also in the process of performing parallel studies on a smaller number of progressed small lymphocytic lymphomas (Richter's syndrome).

Publications:

Van Krieken JHJM, McKeithen TW, Raghoebier S, Medeiros JM, Kluin PhM, Raffeld M. Chromosomal translocation t(14;19) as indicated by bcl-3 rearrangement is a rare phenomenon in non-Hodgkin's lymphomas and chronic leukemias. A molecular genetic study of 176 cases. Leukemia 1990;4:811-14.

Yano T, Longo D, Jaffe ES, Raffeld M. C-myc rearrangements in progressed follicular lymphomas. Blood (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09366-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Polymerase Chain Reaction Systems for T/B Clonality in Lymphoid Neoplasms

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

PI:	T. Greiner	Clinical Associate	LP NCI
OTHER:	E. Jaffe	Chief, Hematopathology Section	LP NCI
	M. Raffeld	Medical Officer	LP NCI
	D. Kingma	Clinical Associate	LP NCI

COOPERATING UNITS *(if any)*

Fred Dick, M.D., University of Iowa, Department of Pathology
Randy Gascoyne, M.D., British Columbia Cancer Center

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	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.)*

The overall goal of this project is to utilize the polymerase chain reaction (PCR) system to identify clonal gene rearrangements in lymphoid neoplasms. The purposes include the following: detect clonality to support diagnostic work of cases, investigate and characterize lymphoid diseases, and provide sensitive methods to follow patients.

Previous work in the section to identify clonal gene rearrangements has been primarily based on using Southern blot technology. However, the sensitivity is low, the methodology requires 1-2 weeks for completion, and can not be performed on DNA extracted from paraffin embedded tissue. Methods have been described using the polymerase chain reaction to amplify clonal rearrangements in both T and B cell lineages. The section has much experience performing PCR for bcl-2 in follicular lymphomas as a method to identify lymphoid clones. No PCR work has previously been done in the section with the T cell system prior to this report.

T cell gene rearrangements can now be amplified from both fresh tissue and paraffin embedded tissue, including tumors such as acute lymphocytic leukemia, peripheral T cell lymphoma, mycosis fungoides, gamma delta lymphomas, subcutaneous T cell lymphomas, and lymphocytic and large cell lymphomas. A high resolution system using denaturing gradient gel electrophoresis has been adapted to follow the unique gene rearrangement for each patient's tumor. B cell immunoglobulin gene rearrangements can be amplified and this is being used to study the lymphocyte predominant form of Hodgkin's disease. This work provides the basis to do PCR *in situ* on paraffin tissue sections to identify specific cell types involved.

Major Findings:

Dr. Greiner, during prior work at the University of Iowa, had developed PCR primers to amplify some T cell receptor gamma gene rearrangements in acute lymphocytic leukemia and T cell lymphoma. Gene rearrangements were sequenced, and patient specific primers were developed to detect minimal residual disease in follow-up bone marrow samples. In addition, PCR amplification methods to rapidly amplify paraffin embedded tissue were developed.

Primers have been designed and shown to amplify the whole spectrum of T cell receptor gamma rearrangements. Specific genes used in the rearrangements can be identified and there is excellent correlation with Southern blot results.

A second accomplishment has been the ability to amplify T cell gamma gene rearrangements from paraffin embedded tissues. This provides a significant tool to the study of lymphoid neoplasms which have no fresh tissue available for Southern blots. This will be especially applicable for the lymphomas that arise in the skin, or extranodal cases, where routinely pathologists fix entire specimens in formalin. The technique can be performed in two days, adding a rapid time improvement.

Denaturing gradient gel electrophoresis has been adapted for the analysis of amplified gene rearrangements to provide superior resolution of genes based on the tumor's unique DNA sequence. This may provide a method to analyze patient biopsies without resorting to sequencing each rearrangement. Correlation with the DNA sequence and T gamma genes used has been done with 10 cases of acute lymphocytic leukemia (Fred Dick, M.D., collaboration). Diseases that are currently being studied include: peripheral T cell lymphoma, mycosis fungoides, gamma delta lymphomas, subcutaneous T cell lymphomas, and lymphocytic and large cell lymphomas; with plans for angioimmunoblastic lymphadenopathy, lymphomatoid papulosis, T gamma lymphoproliferative disease, and others.

The relationship of B cell lymphomas to Hodgkin's disease is being addressed (in collaboration with Randy Gascoyne, M.D.) by using PCR amplification of B cell gene rearrangements. B cell lymphomas arising in AIDS patients will also be studied that have only paraffin tissue available.

With the above accomplishments, work is planned to develop *in situ* PCR for both T and B cell neoplasms to investigate cell types in tumors, probe for residual disease, and study the nature of lymphoma-like diseases.

Publications:

Greiner TC. Polymerase chain reaction: uses and potential applications in cytology. Diagn Cytopathol 1992;8:61-4.

Burgart LJ, Heller MJ, Reznicek MJ, Greiner TC, Teneyck CJ, Robinson RA. Cytomegalovirus detection in bone marrow transplant patients with idiopathic pneumonitis: A clinicopathologic study of the utility of the polymerase chain reaction on open lung biopsy specimen tissue. Am J Clin Pathol 1991;96:572-6.

Heller MJ, Burgart LJ, Teneyck CJ, Anderson MA, Greiner TC, Robinson RA. An efficient method for the extractionof DNA from formalin-fixed, paraffin-embedded tissue by sonication. Biotechniques 1991;11:372-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09372-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Aneuploidy and Cell Cycle Fractions in Benign and Malignant Tumors

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

PI:	M. Stetler-Stevenson	Senior Staff Fellow	LP NCI
OTHER:	M. Kuchnio	Exchange Scientist	LP NCI
	S. Sebers	Medical Technologist	LP NCI
	J. McClanahan	Medical Technologist	LP NCI

COOPERATING UNITS *(if any)*

NIDDKD, Digestive Diseases Branch

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

We have studied the presence of aneuploidy, S phase and hyperdiploid fractions in bladder washings from patients with transitional carcinoma and benign cystitis. We have found apparent false aneuploid populations in bladder washings from patients with benign cystitis. We have investigated the source of this apparent aneuploid population. We have studied DNA dye saturation kinetics and examined the "NIM" or nuclear isolation method vs. the Vindelov method and have found the Vindelov to be superior. We are currently using monoclonal antibodies to focus on the urothelium and to eliminate from our analysis the reactive cells which may have different nuclear characteristics that lead to differential staining and false aneuploidy. Also, fixatives are being examined to determine the best method to preserve cells for transport to a reference laboratory or until analysis can be performed. Ethanol appears to provide superior coefficients of variation (C.V. indicates quality of analysis and sensitivity of detection of aneuploid populations) compared to methanol and paraformaldehyde. We are still investigating modifications of our ethanol fixation method to improve coefficients of variation. We are also studying the presence of aneuploidy, S phase and hyperdiploid fractions in gastrinoma tissues from 60 patients and correlating these results with clinical prognosis. We developed a method for DNA content analysis from paraffin embedded tissues for this purpose.

Major Findings:

1. False aneuploid populations can be observed in bladder washings from patients with benign cystitis.

2. Ethanol fixatives appear to be superior for preserving cells for DNA analysis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09373-01 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

TIMP-1 Expression by Normal Lymphocytes and in Lymphoid Neoplasms

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

PI:	M. Stetler-Stevenson	Senior Staff Fellow	LP NCI
OTHER:	W. Stetler-Stevenson	Medical Officer	LP NCI
	J. Wang	General Fellow	LP NCI
	K. Ptaszynski	Visiting Fellow	LP NCI
	S. Sebers	Medical Technologist	LP NCI
	J. McClanahan	Medical Technologist	LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have observed the expression of TIMP-1 at the RNA level in hyperplastic tonsils, activated T-cells and some Burkitt cell lines. TIMP-1 was not expressed by numerous T-cell lines or B-cell lines derived from low-grade lymphomas. We have demonstrated secretion of TIMP-1 protein by these Burkitt cell lines and observed that TIMP-1 expression correlates with cell proliferation rates. We are currently studying the effect of TIMP-1 on the kinetics of cell proliferation in the Burkitt cell lines as well as the aggressiveness of these lines in nude mice. We are undertaking a study of the expression of TIMP-1 in aggressive and low-grade B-cell neoplasms using antibodies against TIMP-1.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09144-08 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Identification of Proteins Binding to c-myc Regulatory Sequences

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

PI:	D. Levens	Chief, Gene Regulation Section	LP NCI
OTHER:	R. Duncan	IRTA Fellow	LP NCI
	E. Alesse	Visiting Fellow	LP NCI
	E. Michelotti	Biotechnology Fellow	LP NCI

COOPERATING UNITS *(if any)*

Dr. M. Zajac-Kaye, Medicine Branch, DCT, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.25	3.75	1.5

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☐ (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) 1.5 kb upstream of promoter P1 resides a cell-type and differentiation specific positive cis-element. A novel protein binding to this element has been purified and subjected to proteolytic degradation and partial sequence determination. The information thus obtained allowed the cloning of a gene encoding a novel DNA binding protein. This protein possesses a structure comprised of alternating amphiphathic helices (five) and repeating units (four). The minimal sequence specific DNA binding domain is composed of two helices and two repeats. Analysis of the expression of this factor reveal it to be tissue specific and highly regulated. Functional characterization of the protein is ongoing.

2) 100-150 bp upstream of P1 a complex set of trans-factors binds to a cytidine-rich element repeated five times. Some of the factors which interact with this element possess the ability to recognize specific single-stranded sequences. One of the pyrimidine-rich strand binding proteins is hnRNP protein K. Importantly, the activity of the cis-element *in vitro* can be reduced by inclusion of specific single-stranded oligonucleotide competitors thereby indicating the potential for a molecule with hnRNP K-like properties to regulate c-myc. Additional proteins interact with the purine strand. The purine strand binding factors possess several unusual properties which correlate well with the activity of this element in *in vitro* transcription systems. The purification of these factors with an aim of cloning their genes is in progress.

3) Approximately 1 kb downstream of c-myc promoter P1 is an element which has been found to be mutated frequently in Burkitt lymphoma. Several proteins have been shown by cross-linking or binding with SDS-PAGE purified and renatured polypeptides to interact with this element. Some of these proteins possess unusual properties which suggest interesting regulatory mechanisms for controlling c-myc expression. The purification and characterization of these proteins as well as functional characterization of their binding sites are the topics of current studies.

Major Findings:

1) A highly regulated protein possessing a novel DNA binding motif interacts with a far upstream stimulatory element of the human c-myc gene.

2) Multiple factors, including proteins recognizing specific single-stranded sequences interact with a positive element 100-150 bp upstream of P1; suggestive evidence exists that this element can function while in single-stranded conformation.

3) Multiple proteins interact with a site frequently mutated in Burkitt lymphoma within intron 1 of the c-myc gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09168-05 LP

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Analysis of a Multiprotein Complex Interacting with the Gibbon Ape Leukemia

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

PI:	D. Levens	Chief, Gene Regulation Section	LP NCI
OTHER:	K. Gardner	Medical Staff Fellow	LP NCI
	H. Yasui	Guest Researcher	LP NCI
	T. Davis-Smyth	Biotechnology Fellow	LP NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Pathology

SECTION: Gene Regulation Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component binds with reduced specificity to DNA, but upon forming a complex with the first component, confers greatly enhanced power to discriminate between different sequences. These proteins are distinct and separable from fos/jun (AP1). However, a minor complex is present in MLA 144 which contains a fos-related antigen (FRA). The two components of the major complex can be independently activated in a cell-line specific manner.

The first component of the complex has been identified as a modified form of the jun-d protein. The modification, thus far detected only in T-cells, causes the mobility of the jun-d to increase both in SDS-polyacrylamide gels and in DNA binding electrophoretic mobility shift assays. The nature of the modification is under investigation. In addition, it appears that a population of jun-d exists in T-cells which is relatively inactive with the GALV-AP1 site. Modified jun-d, but not recombinant jun-d interacts with a second component, a protein of approximately 20,000 MW, which augments binding to AP1 sites. Importantly this second protein, termed activator, may play an important role in T-cell activation.

Because binding of multiple distinct complexes to a single cis-element is a common theme recurring in gene regulation, it is important to determine which factors are actually bound and therefore used at a given site *in vivo*. To begin to address this issue, we are attempting to develop methods for enriching specific cis-elements from chromatin, retaining factors bound *in vivo*, to determine which proteins are present in specific complexes. Such a method would prove a powerful adjunct to *in vivo* footprinting studies, which demonstrate *in vivo* occupancy at a given site and transfection studies which demonstrate a potential physiological role for a cis-element and a respective trans-factor, but do not prove which of multiple proteins actually function under normal and pathologic conditions.

Major Findings:

1) The core factor is a modified form of jun-d.

2) jun-d pre-exists in resting T-cells but requires modification to interact with the activator factor.

3) The activator may be capable of contributing to the activity of a variety of cis-elements.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09170-05 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Identification of Genes Regulating the Development of Embryonic Limb Buds

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

PI:	S. Mackem	Expert	LP NCI
OTHER:	M. Ranson	Visiting Fellow	LP NCI
	S. Aguanno	Visiting Fellow	LP NCI

COOPERATING UNITS *(if any)*

K. Mahon, Lab. of Mammalian Genes and Development, NICHD; S. Hughes, FCRDC; K. Schughart, Max Planck Inst. of Immunol., Freiburg, Germany; C. Tickle, Dept. of Anatomy, Univ. College of London, U.K. (research collaborators)

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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, programmed cell death, etc., are also recapitulated in a pathologic manner during oncogenesis. Chick limb development is an attractive system for studying the molecular basis of pattern formation because critical events at the level of tissue/cellular interactions involved in pattern formation have been well characterized and appear to be very similar to those in mammalian systems, and this system is readily amenable to biochemical and molecular analysis as well as microsurgical manipulation.

It is the aim of this long-term project to isolate genes that regulate morphogenesis in the chick embryo limb bud. Two general approaches are being developed: 1) the generation of subtracted cDNA libraries enriched for potential regulatory and induced genes; and 2) the identification of related/new members of conserved gene families that have been implicated in developmental regulatory processes in other systems. Currently, two new members of the homeobox gene family have been identified which are expressed predominantly in limb buds during early development. These genes each show spatially restricted expression domains within the limb bud that suggest roles in regulating pattern formation along the anterior-position (A-P) and proximodistal (P-D) axes of the limb. One of these homeobox genes also displays expression differences between wing and leg buds, indicating it may also regulate the determination of limb-type identity. Studies are underway to elucidate the function of these regulatory genes, using both molecular genetic and biochemical approaches.

Major Findings:

A number of oriented cDNA libraries have been generated from different, selected limb bud mRNA populations, for use in both general screening and for performing library-based subtractive hybridizations to enrich for genes involved in establishing morphologic patterns and in regulating the pattern differences between wing and leg that constitute limb-type identity. These include early stage (17/18) wing, leg and late stage (21/22) wing, leg libraries. These different stages were chosen as likely to represent times at which signals regulating A-P and P-D patterning are just beginning to be expressed ("early") and times at which apparent morphogenetic gradients are well established ('late'), but prior to the onset of frank tissue differentiation. Currently, subtracted libraries enriched for wing- and for leg-specific sequences have been generated and screening with wing- and leg-enriched cDNA probes is underway to isolate genes regulating differences in wing/leg pattern formation.

We have used a PCR-based approach employing degenerate oligonucleotide primers for amplification, and subsequent subcloning and sequencing, to identify homeobox genes that are expressed in limb bud mRNA populations. At least 18 different homeobox genes appear to be expressed in chick embryo limb buds. Some of these include genes that have previously been characterized in other vertebrate systems and are known to be expressed in developing and/or regenerating limbs. Several genes appear to be new members of the homeobox family in vertebrates and two of these are selectively expressed predominantly in limb buds during early development. These genes are novel non-Antennapedia homeobox genes with homeodomain sequences of some similarity to *Drosophila Abd-B* (*Ghox 4.7*) and *Dll* (L5) respectively, and we have extensively analyzed their spatiotemporal expression domains during development using *in situ* hybridization techniques on both sectioned embryos and on whole mount embryos.

Ghox 4.7 has been named such because it is homologous to the murine 4.7 *Hox 4* cluster gene. We have found that this gene is expressed in a highly posteriorly restricted domain of the early limb bud, suggesting a role in patterning along the A-P axis. Several laboratories 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 (retinoid treatment, polarizing grafts anteriorly) will result in ectopic mirror-image duplication of expression of several *Hox 4* cluster genes in the anterior limb bud, also supporting the notion that these posteriorly expressed genes regulate the A-P pattern. Using whole mount *in situ* hybridization with several of these chick *Hox 4* cluster genes, we have found that the very posteriorly restricted *Hox 4* genes also display quantitative and qualitative expression differences between wing and leg buds in the chick embryo. They are expressed at a higher level and over a broader P-D zone in the posterior part of the wing than the leg bud. These differences may be related to modification of the avian wing from the general tetrapod limb pattern for flight, since some of the posterior digital elements of the wing are enlarged and predominate its structure. Hence, the limb-type differences in chick *Hox 4* expression may also reflect a role in regulating A-P pattern.

The second gene (L5) has a highly restricted expression domain along the P-D axis, 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 limb outgrowth. At later stages (st 26-28), the expression is localized more proximally, and is restricted to the region of the anterior distal zeugopod (radius or tibia) and proximal autopod (carpals or tarsals). This type of expression is consistent with the known progressive determination of structures along the P-D axis in a proximal to distal sequence and suggests a role for L5 in the determination of positional identity (for example of the wrist/ankle) along the P-D axis. Microsurgical manipulations (apical ridge excisions/grafts; retinoid treatment) are currently underway to analyze the expression pattern of this gene when the developmental program (pattern) is experimentally altered. Interestingly, L5 is also expressed in two other locations in the very early embryo; the anlage of the pineal gland, and in the portion of the notochord adjacent to paraxial mesoderm that has not yet become segmented into somites (which recedes caudally as development proceeds).

Long-term experiments to determine the function of these genes are currently underway, and include characterizing the effects of ectopic overexpression as well as ablation of expression of these genes using transgenic technology in mice, and avian retroviral expression vectors in chick embryos. These genetic analyses are already in progress to analyze the function of the *Ghox 4.7* 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 *Hox 2.2* promoter) in order to target (and restrict) overexpression of *Ghox 4.7* to the developing limb bud.

Biochemical approaches are also being employed to identify downstream "targets" that are regulated by *Ghox 4.7* during limb morphogenesis. The cis DNA sequence elements to which the *Ghox 4.7* protein binds have been identified, and are unlike the AT-rich sequences that many other homeobox genes recognize. Specific antibodies against the Ghox 4.7 protein have been raised, and these will be useful in the isolation of *in vitro* and also *in vivo* complexes formed between the *Ghox 4.7* protein and genomic DNA for the purpose of identifying downstream target genes that *Ghox 4.7* regulates.

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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09171-09 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

The Regulation of Lymphocyte Proliferation

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
OTHER:	K. Smith	Microbiologist	LP NCI
	J. Gray	Fogarty Fellow	LP NCI
	P. Davis	Technician	LP NCI
	P. Jensen	Technician	

COOPERATING UNITS *(if any)*

Ulrich Siebenlist, Ph.D., Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. Primary sequence analysis has revealed that 3 early induced genes code for membrane associated proteins. Activation-induced changes in cell surface proteins resulting from a primary stimulus play a particularly important role in regulating downstream proliferative and differentiative responses. Important events mediated at the cell surface include the binding of soluble factors and interactions with other cells and extracellular matrix. 237 encodes a hematopoietic cell-specific, 23 kDalton type 2 membrane surface protein. 237 belongs to the C-type lectin superfamily of proteins. Other known C-type lectins on lymphoid cells are signal-transducing molecules. 237 is unique in that its expression is induced in activated cells. 276 encodes a surface protein that contains 7 membrane-spanning regions characteristic of signal-transducing receptors that couple to G proteins. 276 also contains at the amino terminus, EGF repeats that are thought to be involved in protein-protein interactions, and thus are often found on receptors or their ligands. 154 encodes a 158 amino acid peptide that contains a hydrophobic leader sequence and no other putative transmembrane region. 154 does not appear to be secreted but is membrane-associated, possibly predominantly with the endoplasmic reticulum.

Major Findings:

We are investigating the consequences of mitogen mediated signals to T cells by isolating and characterizing genes that constitute the immediate early transcriptional response to these events. We have isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. Selected clones have been characterized with regard to primary sequence, analyses of the encoded protein with regard to biochemical properties and subcellular location, and biological parameters of expression. Three clones will be discussed here (237, 154, and 276) that appear to be membrane-associated. 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.

237 encodes an early activation-specific gene in lymphocytes that encodes a 199 amino acid protein that has a single internal hydrophobic stretch of amino acids characteristic of type 2 membrane proteins which orient proteins across the membrane with the carboxy terminus on the exterior surface of the cell. A rabbit polyclonal anti-peptide antibody generated to a carboxy terminal segment immunoprecipitates a 23 kDalton protein from Cos cells transfected with a 237 expression construct. A 23-26 kD diffuse protein band is immunoprecipitated from cell surface iodinated, activated human peripheral blood T cells. 237 protein is also present on resting T cells, but in approximately 10-20 fold lower amounts. Data base comparisons reveal that 237 belongs to the calcium-dependent (C-type) lectin superfamily. Other surface molecules on lymphoid cells which have C-type lectin structures include CD 23 (the low affinity IgE receptor) and CD 72 on B cells, and three separate families of proteins (NKRP, NKG2, and Ly49) expressed on NK cells. The above molecules have in common the property of mediating signal transduction following binding by a specific monoclonal antibody. 237 is unique among C-type lectins in that expression is induced in lymphoid cells by antigens or mitogens, suggesting a potential function in modulating growth and/or differentiative signals at the cell surface associated with the activation process.

Sequence analysis of the 276 gene is nearly complete, although the precise amino terminus of the protein has not been determined. Translation of the nucleotide sequence reveals a protein with "EGF repeats" at the amino end of the protein and 7 potential membrane-spanning regions throughout the rest of the molecule. The membrane-spanning regions are characteristic of receptors that couple through GTP-binding proteins during signal transduction. EGF repeats are thought to be a particular motif involved in protein-protein interactions. Thus, they are often conserved in cell surface receptors or their ligands. Thus, 276 appears almost certainly to be a signal-transducing receptor.

154 encodes a 158 amino acid peptide with a hydrophobic leader sequence but no other transmembrane domain. Rabbit polyclonal anti-peptide antibodies and several murine monoclonal antibodies have been generated to the 154 protein. The 154 protein does not appear to be secreted as might have been predicted from the structure. Cos cells transfected with a 154 expression vector construct

display 154 protein associated with the endoplasmic reticulum (ER). The subcellular location of the 154 protein in activated T cells is being investigated. Although one prediction might be that 154 associates with another protein in the ER in transit to the cell surface, there is no positive evidence from FACS analysis to support this possibility. Alternatively, 154 may be a resident ER protein involved in potential functions such as protein synthesis, protein turnover, or protein oligomerization.

Publications:

Bours V, Burd PR, Brown K, Villalobos J, Park S, Ryseck R-P, Bravo R, Kelly K, Siebenlist U. A novel mitogen-inducible gene product related to p50/p105/NF-κB participates in transactivation through a κB site. Molec Cell Biol 1992;12:685-95.

Kelly K, Davis P, Mitsuya H, Irving S, Wright J, Grassmann R, Fleckenstein B, Wano Y, Green W, Siebenlist U. A high proportion of early response genes are constitutively activated in T cells by HTLV I. Oncogene (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09357-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

RAI-1: A Mitogen-Inducible RAS-Related Protein

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
OTHER:	J. Maguire	Senior Staff Fellow	LP NCI
	P. Davis	Technician	LP NCI
	P. Jensen	Technician	LP NCI
	T. Santaros	Guest Researcher	LP NCI

COOPERATING UNITS *(if any)*

Ulrich Siebenlist, Ph.D., Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.8	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A gene has been isolated from an activated T cell library that appears to encode a novel low molecular weight GTP-binding protein that demonstrates approximately 20 percent homology to c-Ha-ras. This gene has been designated RAI-1 for transcriptionally induced ras-related protein. RAI-1 codes for a 35 kdalton phosphoprotein that contains concensus GTP binding elements, although the G-3 element is imperfect. RAI-1 has been shown to preferentially bind GTP over ATP. RAI-1 differs from c-ras proteins by the presence of amino and carboxy-terminal extensions and the absence of an acceptor site for isoprenylation. RAI-1 message is transiently expressed in mitogen-activated cells of various lineages. RAI-1 appears to be associated with the endoplasmic reticulum, most likely through an association at the cytoplasmic face. The GTP-binding property of RAI-1 in addition to its phosphorylation (which can often be regulatory for function) suggest the possibility that RAI-1 is a signal transducing protein associated with communication between the lumen of the ER and the cytoplasm.

Major Findings:

We are investigating the consequences of mitogen mediated signals to T cells by isolating and characterizing genes that constitute the immediated early transcriptional response to these events. We have isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. One such clone, pAT 270, encodes a 32 kd protein that shows approximately 20-25 percent homology to proteins of the ras/rap/ral family. Therefore, we have renamed pAT 270 RAI-1 for transcriptionally induced, ras-related protein. RAI-1 shows the presence of guanine nucleotide binding concensus elements, although the G-3 region is imperfect. *In vivo* cross-linking experiments using radiolabeled GTP and ATP have demonstrated that RAI-1 preferentially binds GTP. Whether RAI-1 has intrinsic GTPase activity is currently being investigated.

Polyclonal antibodies to predicted peptides and monoclonal antibodies to bacterially-produced recombinant protein have been generated and characterized. Immunoprecipitation studies of extracts from mitogen-stimulated peripheral blood T cells reveal a diffuse band of 35 kd that demonstrates peak synthesis in parallel with mRNA expression at approximately four hours following stimulation. The half-life of the protein is approximately 2 hours. Peak accumulation of RAI-1 occurs in the G1 phase of the cell cycle. RAI-1 is phosphorylated as shown by the decreased migration of phosphatase-treated protein and by reactivity with anti-phosphotyrosine antibodies. Immunofluorescence studies show that RAI-1 is associated with the endoplasmic reticulum. Because RAI-1 does not include any potential membrane spanning sequence, it is likely that RAI-1 is associated with the external face of the ER. Coupled with the GTP-binding activity of RAI-1, it seems possible that RAI-1 may play a role in signal transduction through the endoplasmic reticulum.

The conservation of genes from mammalian to simple eucaryotic organisms provides a powerful genetic tool for investigating function. Human RAI-1 cDNA has been used to isolate a cross-reactive genomic clone from yeast which is currently being analyzed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09358-02 LP

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Characterization of a Mitogen-Inducible Tyrosine Phosphatase, CAP-1

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
OTHER:	P. Rohan	Biotechnology Fellow	LP NCI
	P. Davis	Technician	LP NCI
	C. Moskaluk	Clinical Associate (MSF)	LP NCI
	P. Jensen	Technician	LP NCI

COOPERATING UNITS *(if any)*

Ulrich Siebenlist, Ph.D., Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A transiently-expressed mitogen-inducible gene has been isolated from an activated T cell cDNA library that appears to encode a phosphotyrosine phosphatase (PTPase). We have designated this gene CAP-1 for cellular activation-related phosphatase. DNA sequence analysis has revealed a 311 amino acid peptide that contains a concensus tyrosine phosphatase active site at the carboxy terminus, but is otherwise unique in sequence, possibly defining a new class of PTPase. No transmembrane domain is apparent, suggesting that CAP-1 belongs to the soluble class of PTPases. CAP-1 mRNA is expressed in mitogen-activated or growing cells of various lineages. Immunoprecipitation analyses in activated T cells demonstrate a 32 kD protein which is induced and maximally expressed during the G1 phase of the cell cycle, and has an apparent half-life of 30 minutes. Three additional non-covalently associated proteins co-precipitate with CAP-1 and may represent functional modulators of CAP-1 activity. As tyrosine phosphorylation is a major control mechanism in growth, the possible role of CAP-1 in regulating cell cycle progression is being addressed.

Major Findings:

We are investigating the consequences of mitogen mediated signals to T cells by isolating and characterizing genes that constitute the immediate early transcriptional response to these events. We have isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. One such clone, formerly designated pAT 120, contains within the encoded longest open reading frame a concensus sequence for the active enzymatic site of phosphotyrosine phosphatases. Therefore, we have now renamed pAT 120 as CAP-1 (for cellular activation-related phosphatase). CAP-1 message codes for an approximately 32 kd protein that is cysteine-rich. The expected size for CAP-1 protein has been confirmed by *in vitro* transcription and translation. No obvious transmembrane segments are apparent. CAP-1 shows no remarkable sequence homology outside the active site to a variety of PTPases that have been previously described, thus defining a new structural class of PTPase. Sequence including and immediately adjacent to the active enzymatic site of CAP-1 is most closely related to the VH-1 phosphatase which has dual specificity for both tyrosine and threonine/serine. The CAP-1 locus has been mapped to the short arm of human chromosome 2.

In order to characterize CAP-1 protein *in situ*, a variety of antibodies have been raised against peptides to the predicted protein sequence or to bacterially-produced, recombinant CAP-1 protein. Three separate antipeptide antibodies and three antibodies to various regions of the recombinant protein demonstrate reactivity to native human protein as assayed by Western blots and/or immunoprecipitation. A protein of the predicted size, 32 kD, is observed in Cos cells transfected with a CAP-1 expression construct and in mitogen-stimulated peripheral blood T cells. Kinetic analyses of protein expression in mitogen-stimulated PBL demonstrate maximum relative expression parallel with mRNA levels within the first 4 hours following stimulation. Total accumulated expression peaks in mid to late G1, approximately 12 to 18 hours after stimulation. The half-life of the CAP-1 protein is approximately 30 minutes. Immunoprecipitation studies have revealed three additional, noncovalently linked proteins (20.5 kD, 38 kD, and 85 kD) that are coprecipitated with CAP-1. Such proteins may be CAP-1 associated proteins and therefore potential modulators of function, or alternatively, immunologically cross-reactive and possibly structurally related. In addition to further characterizing the expression profile of native CAP-1 protein and its associations, antibodies will be used to establish subcellular localization of CAP-1.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBDC, NCI
October 1, 1991 through September 30, 1992

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 five separate, though frequently interacting, areas. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 2,000 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year are as follows:

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases (Dr. Stephen Katz):

We have continued our studies of the immunological functions of cells of the epidermis. During the past year we have been investigating 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. We have found that increased amounts of IL-1β mRNA can be detected as early as 15 min. after hapten painting. This IL-1β mRNA comes mainly from Langerhans cells. We have investigated the potential role of IL-1β in inducing some of the activation events in Langerhans cells and have found that IL-1β may mimic the affects of allergens on Langerhans cells on a molecular level as well as a biological level. In addition, we have found keratinocytes produce IL-10 protein both constitutively and after stimulation with haptens such as DNCB. This IL-10 may play a critical role in the ultimate activation of Th2 cells in that it abrogates Th1 proliferation. Further in vivo and in vitro studies will determine whether the IL-10 may play a role in the T cell response to contact allergens. The studies of T cell activation by human Langerhans cells are continuing. We are now ready to investigate the immunological functions of LC in patients with AIDS.

Regulation of Cutaneous Accessory Cell Activity in Health and Disease (Dr. Mark Udey):

A major focus of this laboratory has been the study of costimulatory molecules important in Langerhans cell-T cell activation. Studies designed to identify important adhesion (or costimulatory) molecules on LC are ongoing. mRNA encloding the murine homologue of the B cell activation antigen B7 (BB1), a costimulatory molecule thought to be important in the activation of Th1 cells, has been reverse transcribed, amplified using the polymerase chain reaction and detected in samples of epidermal cell RNA by autoradiography after fluid-phase hybridization with complementary radiolabelled synthetic oligonucleotides. Quantitative studies have revealed that cultured LC express

100-1000x more B7 mRNA than fresh LC or keratinocytes (KC) and 10-100x more B7 mRNA than the reference cell line CH-1. Cultured murine LC also provide costimulatory activity for human T cells in a xenogeneic assay that we believe measures B7 functional activity. Expression of B7 by cultured LC may be largely responsible for the ability of LC to activate unprimed T cells. Very recent studies may also provide insight into mechanisms that promote the localization of LC in epidermis. We have determined that LC express cadherins, that LC adhere to E-cadherin expressing cells in vitro, and that anti-E cadherin mAb abrogates LC-KC binding. E-cadherin expression by LC reflects endogenous synthesis since LC also contain E-cadherin mRNA. The role that cadherins play in LC biology and in the localization of other leukocytes in epithelia is under investigation.

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. Pemphigus vulgaris is an acantholytic blistering disease wherin patients have autoantibodies to epidermal cell surface molecules. These have been very well characterized - the antigen is related to desmoglein I, which is a cadherin. The full length coding sequence for PV antigen has been cloned. The deduced amino acid sequence of this antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules and is closely related to the PF antigen. PV patients have antibodies against the amino-terminal domain of this molecule, an area thought to be important in its adhesion function, and these antibodies can cause loss of adhesion of epidermal cells in an animal model of disease.

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 being expanded. The effectiveness of oral isotretinoin as a chemopreventive agent in patients with high rates of skin cancer formation has been demonstrated. Patients are now maintained on long-term isotretinoin for chemoprevention. A phase I/II study of recombinant human interferon gamma for basal cell carcinoma is ongoing. All 6 tumors treated to date have become smaller; 2 underwent complete histological regression.

Dr. DiGiovanna is also actively collaborating with several groups to determine whether genetic linkage exists between certain heritable skin diseases and gene clusters. In collaboration with Allen Bale at Yale, he has identified a mutation in a tumor suppressor gene that is the probable cause of the nevoid basal cell carcinoma (Gorlin's) syndrome and demonstrated tight linkage of the syndrome gene to this region. His collaborative study with Sherri Bale and Peter Steinert (Laboratory of Skin Biology - NIAMS), has successfully identified linkage of the epidermolytic hyperkeratosis gene to the type II keratin gene cluster on chromosome 12q. These studies are continuing.

Studies of DNA Repair in Normal Human Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders (Dr. Jay Robbins):

Studies in this laboratory are designed to elucidate the role of DNA repair processes in carcinogenesis and in neurodegeneration. In collaboration with Dr. Katherine K. Sanford and Dr. Ram Parshad (Department of Pathology, Howard University College of Medicine), we have been developing cytogenetic tests which have the potential of showing very large differences in radiation-induced chromosomal aberrations between normal and Alzheimer disease (AD) cells. These studies will also hopefully help us better understand the pathogenesis of this disease. We have performed irradiation of normal and AD fibroblast strains in either the G_1- or G_2-phases of the cell cycle. Caffeine, a strong inhibitor of DNA repair during S phase, was added in some experiments. We have tested 4 AD and 4 normal fibroblast strains (2 familial; 2 sporadic) in the G_1 test and 2 of the AD and 2 of the normal strains in the G_2 test. In the G_1 test there were significant differences only in the presence of caffeine between the AD and normal cells in the total number of chromatid gaps and breaks per 100 metaphase cells. These G_1 results indicate that the normal cells repair the light-induced damage in the presence or absence of caffeine, while the AD cells cannot repair the damage in the presence of caffeine. In the G_2 test, the 2 normal strains had β-cytosine arabinoside (ara-C)-induced increases of the light-induced chromatid gaps and breaks of 19 and 22 per 100 metaphase cells, while the 2 AD strains had increases of only 5 and 3. One interpretation of these results is that the normal strains attempt to repair the light-induced damage in G_2 by making incisions at the sites of damage but are unable to seal the resulting gaps because of the ara-C, while the AD cells are deficient in performing the initial repair incision at sites of light-induced damage and thus have no gaps to be blocked by the ara-C. We are attempting to adopt the G_1 and G_2 tests to fibroblasts in plastic flasks (rather than in Leighton tubes) and to lymphoblast cell lines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03638-23 D

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Studies of DNA Repair in Human Degenerative Diseases

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

PI: J. H. Robbins, M.D., Dermatology Branch, DCBDC, NCI

OTHER: V. A. Bohr, M.D., Senior Investigator, LMPH, DCT, NCI
K. S. Sanford, Ph.D., Senior Investigator, LCMB, DCE, NCI
L. Seguin, Ph.D., Consultant, D, DCBDC, NCI
R. E. Tarone, Ph.D., Mathematical Statistician, BB, DCE, NCI

COOPERATING UNITS *(if any)*

Laboratory of Molecular Pharmacology, DCT, NCI; Laboratory of Cellular and Molecular Biology, DCE, NCI; Biostatistics Branch, DCCP, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
3.2	1.3	1.9

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.)*

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.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

In contrast to the conventional study of repair in the genome overall, in collaboration with Drs. Vilhelm A. Bohr and Michele K. Evans, we are studying the repair of ultraviolet radiation (UV)-induced cyclobutane pyrimidine dimers in an active housekeeping gene [the dihydrofolate reductase (DHFR) gene], the inactive delta-globin gene, and in the active c-myc protooncogene. In this procedure isolated DNA from fibroblasts is restricted, separated from replicated DNA, nicked at dimers with T-4 endonuclease V, electrophoresed in alkaline agarose, and subjected to Southern hybridization. We have studied these genes in normal and XP group-A, C, D, and F fibroblast strains. We have found that the normal strain repaired in 24 h approximately 80% of the dimers in its DHFR gene, 95% in its c-myc gene, and 65% in its delta-globin gene. It repaired 88% of the dimers in the transcribed strand of its DHFR gene but only 66% in the nontranscribed strand. Thus, our normal strain appears to repair different genes at different rates and to repair the transcribing DHFR strand faster than the nontranscribing strand. The two group-C strains removed approximately 45-60% of the dimers from their DHFR and C-myc genes. The two group-C strains repaired approximately 65-75% of the dimers in the transcribed strand of the DHFR gene but much less in the nontranscribed strand. One group-C strain removed 40% of the dimers from the delta-globin gene, while the other strain removed <20%. Thus, these two group-C strains may differ in their repair of the delta-globin gene. In the group-A, D, and F strains very little repair (<20%) was found in the three genes. Since the group-F strain, in comparison with the group-A, C, and D strains we studied, is known to have the highest post-UV survival and to be from the patient with the latest age of onset of skin cancer and with the least likely chance of developing XP neurologic disease, we can conclude that the repair of dimers in active genes is not determinative for cell survival or for these clinical manifestions of XP.

We have been collaborating with Dr. Katherine K. Sanford and Dr. Ram Parshad (Department of Pathology, Howard University College of Medicine), in the development of cytogenetic tests which have the potential of showing very large differences in radiation-induced chromosomal aberrations between normal and Alzheimer disease (AD) cells. We have performed irradiation of normal and AD fibroblast strains in either the G_1- or G_2-phases of the cell cycle with cool-white fluorescent light. X-rays were not used, because they produce a mitotic block in early G_2, but fluorescent light does not perturb the cell cycle. All experiments were conducted in glass Leighton culture tubes, each of which contained one coverslip on which the cells were grown. They were irradiated through the glass tubes in a 37° walk-in incubator. To test for repair of damage inflicted during G_1, cells were exposed to the light for 5 hours and incubated an additional 15 hours, during the last 1 hour of which Colcemid was added. Caffeine, a strong inhibitor of DNA repair during S phase, was added to half the tubes for the 15-hour incubation period. To test for repair of damage inflicted during G_2, cells were exposed for 2 hours to the light; cells entering metaphase from 0.5-1.5 hours after the 2-hour exposure were arrested by Colcemid. B-cytosine arabinoside (ara-C), an inhibitor of the repair replication required for completing DNA excision repair,

was added to half the tubes 10 minutes after the 2-hour light-exposure. We have tested 4 AD and 4 normal fibroblast strains (2 familial; 2 sporadic) in the G_1 test and 2 of the AD and 2 of the normal strains in the G_2 test. In the G_1 test there were no significant differences in the absence of caffeine between the AD and normal cells in their total number of chromatid gaps and breaks per 100 metaphase cells. However, the 4 normal strains had no significant caffeine-induced increases in their fluorescent light-induced chromatid breaks per 100 metaphase cells, the total increases being 0, -3, 1, and 0, while the corresponding values for the 4 AD strains were as follows: 66, 37, 33, and 25. These G_1 results indicate that the normal cells repair the light-induced damage in the presence or absence of caffeine, while the AD cells cannot repair the damage in the presence of caffeine. In the G_2 test, the 2 normal strains had ara-C-induced increases of the light-induced chromatid gaps and breaks of 19 and 22 per 100 metaphase cells, while the 2 AD strains had increases of only 5 and 3. One interpretation of these results is that the normal strains attempt to repair the light-induced damage in G_2 by making incisions at the sites of damage but are unable to seal the resulting gaps because of the ara-C, while the AD cells are deficient in performing the initial repair incision at sites of light-induced damage and thus have no gaps to be blocked by the ara-C. We are attempting to adopt the G_1 and G_2 tests to fibroblasts in plastic flasks (rather than in Leighton tubes) and to lymphoblast cell lines.

All XP patients have abnormally increased frequencies of UV-induced skin cancers. It is known that nucleotide DNA-repair deficient XP patients in complementation groups A and C have abnormally high levels of UV-induced chromosomal aberrations. We have completed our studies on UV-induced chromosomal aberrations in lymphoblast lines from patients with the group-E and XP variant forms of XP. These two forms of XP have entirely different DNA-repair phenotypes. Group-E cells have abnormal levels of UV-induced DNA-repair synthesis and normal postreplication repair, while variant cells have normal UV-induced DNA-repair synthesis and abnormal postreplication repair. We have found that both forms of XP had normal levels of UV-induced chromosomal aberrations. Even when the variant cells were studied in the presence of caffeine, which exacerbates their postreplication repair defect, normal levels of UV-induced chromosomal aberrations were found. Thus, we conclude that abnormally increased levels of UV-induced chromosomal aberrations are not even necessary for XP patients to have UV-induced skin cancer. Either abnormal nucleotide-excision repair, as measured by UV-induced DNA-repair synthesis in XP group-E patients, or abnormal postreplication repair in XP variants is sufficient to cause their UV-induced skin cancer.

We have performed studies to confirm the complementation group assignment of 3 of our unrelated XP patients believed to be in complementation group D. The patient fibroblast strains were labeled with latex beads of one size; the reference group-D strain with beads of another size. After a patient's strain was fused to the reference strain to form multinucleate cells, heterokaryons were identifiable on autoradiograms because they contained beads of both sizes. We found that the heterokaryons formed between the reference group-D strain and each of the 3 additional strains had no more UV-induced unscheduled DNA synthesis than any of the strains when unfused. Thus, we confirmed that the 3 additional XP patients were in group D.

Publications:

Robbins JH, Brumback RA, Mendiones M, et al. Neurological disease in xeroderma pigmentosum: Documentation of a late onset type of the juvenile onset form, Brain 1991;114:1335-1361.

Barrett SF, Robbins JH, Tarone RE, Kraemer KH. Evidence for defective repair of cyclobutane pyrimidine dimers with normal repair of other DNA photoproducts in a transcriptionally active gene transfected into Cockayne syndrome cells, Mut Res 1991;255:281-291.

Robbins JH. Xeroderma pigmentosum complementation group H is withdrawn and reassigned to group D. [Letter to the Editor]. Hum Genet 1991;88:242.

Sequin LR, Ganges MB, Tarone RE, Robbins JH. Skin cancer and chromosomal aberrations induced by ultraviolet radiation: Evidence for lack of correlation in xeroderma pigmentosum variant and group E patients, Cancer Genet Cytogenet 1992;60 (in press).

Robbins JH, Brumback RA, Moshell AN. Clinically asymptomatic xeroderma pigmentosum neurological disease in an adult: Evidence for a neurodegeneration in later life caused by defective DNA repair, Europ Neur 1992 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03657-18 D

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

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

P.I.: S.I. Katz, Branch Chief, Dermatology Branch, DCBDC, NCI

OTHER: P. Cohen, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI
E. Dugan, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI
A. Blauvelt, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI
A. Enk, Special Volunteer, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS *(if any)*
Dermatology Dept., USUHS, Bethesda

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6	5	1

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

B

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

The major area of study of this laboratory is the role of the epidermis as an immunological organ. We have found that epidermal Langerhans cells are derived from precursor cells in the bone marrow and play an essential role in many of the immunological reactions affecting the skin. We have demonstrated that when murine epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells for allogeneic and autologous T cells compared to freshly prepared Langerhans cells. We have therefore utilized cultured Langerhans cells for the generation of primary immune responses in resting unsensitized T cells. We have demonstrated that when cultured cells are modified with hapten, they can generate primary immune responses. The sensitized T cells thus generated respond preferentially to the same hapten in vitro. Another major focus of this laboratory has been the study of the function of class II MHC bearing keratinocytes which appear in humans and mice during cell-mediated reactions in the skin. Our studies demonstrate that these class II bearing keratinocytes induce specific immunological unresponsiveness in cloned T cells and in vivo in contact sensitization. We have also been studying the early cell and molecular events which occur after hapten painting of skin of nonsensitized mice to identify potentially important Langerhans cell and keratinocyte alterations. In response to hapten painting, activated Langerhans cells appear in vivo and there is a differential upregulation of epidermally-derived cytokine mRNAs. The cellular source of these cytokine mRNAs has been identified using cell depletion studies. We have found that IL-1β is the earliest cytokine to be activated. Furthermore, we have identified that IP-10, MIP-2 and IL-10 are produced by keratinocytes. We have also demonstrated that IL-1β plays a critical role in the "activation" of Langerhans cells.

Project Description

Major Findings:

We have found that when murine epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells for the generation of allogeneic and autologous T cell responses as well as for T cell responses to hapten modified self and for protein antigens. When cultured, these cells express much greater amounts of Class II antigens than do freshly prepared cells. Utilizing these cells in primary immune responses in vitro, we found that the T cells responded preferentially to the hapten to which they were "primed." We have produced T cell lines from these in vitro sensitized cells and virtually all of them produce IL-4 as opposed to IL-2. We have found that during the first few stimulations, the primed T cells produce mainly IL-2, however after multiple stimulations they produce mainly IL-4 and not IL-2. We are currently extending these studies to tumor systems in which there are well-defined tumor-associated antigens.

We are continuing our studies in human beings in which we are attempting to generate primary T cell responses in vitro. We have demonstrated that cultured human Langerhans cells exhibit increased class II MHC molecules and are potent alloantigen presenting cells. We are assessing their ability to present protein antigens and will then attempt to generate primary in vitro responses to viral-or tumor-associated antigens.

During the past two years we have been investigating the very earliest events which occur after skin is exposed to haptens and other chemicals, and have found that within 24 hours there is "activation" of Langerhans cells as demonstrated by their expressing greatly increased amounts of class II MHC on their surfaces, as well as their becoming much more potent antigen presenting cells than are "unstimulated" Langerhans cells. In addition we are assessing changes in epidermis-derived cytokine mRNA levels early in the afferent phase of contact sensitivity. We are using a sensitive reverse transcriptase-PCR-technique to quantitatively compare the patterns of mRNA regulation of the following: class II MHC I-Aα, TNF-α, IL-1α, IL-1β, interferon (IFN)-γ, GM-CSF, IFN-induced protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2). Enhanced LC-derived IL-1β mRNA signals are detected as early as 15 min after skin painting with allergens. While TNF-α, IFN-γ and GM-CSF mRNAs are upregulated after application of allergens, irritant and tolerogens, class II MHC I-Aα, IL-1α, IL-1β, IP-10 and MIP-2 mRNAs are upregulated only after allergen painting. Depletion of specific cell populations demonstrates that Langerhans cells are the primary source of the IL-1β and class II MHC I-Aα mRNA's, keratinocytes are the primary source of the TNF-α, IL-1α, IP-10 and MIP-2, and infiltrating T lymphocytes are the source of the IFN-γ. The studies demonstrate that LC-derived and certain keratinocyte-derived cytokine mRNAs are selectively upregulated by allergens in the very early afferent phase of contact sensitivity. As the earliest manifestation of LC activation is the accummulation of increased amounts of IL-1β mRNA in LC within 15 min after exposure to contact allergens, we assessed the functional role of IL-1β. We found that IL-1β mimics the effects of allergens on LC class II MHC antigen expression, accessory cell activity and mobilization and simulates the effects of contact allergens or LC- and keratinocyte-derived cytokine mRNA signals.

In aggregate, our studies show that IL-1β plays an essential role in the initiation of primary cutaneous responses. (Time devoted to AIDS is 20%).

Publications:

Gaspari A, Katz SI. Induction of in vivo hyporesponsiveness to contact allergens by hapten-modified Ia^+ keratinocytes, J Immunol 1991;147:4155-4161.

Enk A, Katz SI. Early molecular events in the induction phase of contact sensitivity, Proc Nat Acad Science (USA) 1992;89:1398-1402.

Enk A, Katz SI. Identification and induction of keratinocyte-derived IL-10, J Immunol (in press)

Cohen PJ, Katz SI. Cultured human Langerhans cells process and present intact protein antigens, J Invest Derm (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03659-18 D

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Therapy of Skin Cancer, Psoriasis, Disorders of Keratinization and Cystic Acne

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

P.I.: J.J. DiGiovanna, Expert Scientist, Dermatology Branch, DCBDC, NCI

Other: I. Tokar, Registered Nurse, Clinical Center Nursing
K. Kraemer, Senior Investigator, Lab. Molecular Carcinogenesis
Maria Turner, M.D., Medical Officer, Dermatology Branch, DCBDC, NCI
Nicholas Patronas, Radiology Department, Clinical Center
T. Peter Bridge, Senior Investigator and AIDS Clinical Coordinator, Laboratory of Clinical Science, NIMH

COOPERATING UNITS *(if any)*

Sherri Bale, Ph.D., Family Studies Section, EEB, DCE
Peter Steinert, Ph.D., Senior Investigator, Lab. Skin Biology, NIAMS
Allen E. Bale, M.D., Dept. of Genetics, Yale University School of Medicine

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

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 for dermatologic diseases, particularly, skin cancer and the disorders of keratinization. During the last decade over 300 patients studied have established the efficacy and characterized the toxicity of isotretinoin and etretinate in the treatment of a variety of disorders. Patients requiring long-term retinoid therapy continue to be monitored. Skeletal toxicity is an important chronic side effect. The high rate of peripheral skeletal involvement that occurs after chronic etretinate therapy was first identified in these patients. In patients with Darier's disease, we have identified and are characterizing a novel, common, cystic bone abnormality. Peptide T is a synthetic oligopeptide which has been associated with improvement in HIV related psoriasiform eruptions. After an initial clinical trial of intranasal Peptide T for psoriasis showed minimal efficacy, a topical study was begun. We are expanding our studies directed at skin cancer treatment and prevention . We demonstrated the effectiveness of oral iostretinoin as a chemopreventive agent in patients with high rates of skin cancer formation. These patients are now maintained on long-term isotretinoin for chemoprevention. A phase I/II study of recombinant human interferon gamma for basal cell carcinoma is ongoing. All 6 tumors treated to date have become smaller; 2 underwent complete histological regression. In collaboration with Allen Bale we have identified that a mutation in a tumor suppressor gene is the probable cause of the nevoid basal cell carcinoma (Gorlin's) syndrome and demonstrated tight linkage of the syndrome gene to this region. Our collaborative study with Sherri Bale and Peter Steinert, has successfully identified linkage of the epidermolytic hyperkeratosis gene to the type II keratin gene cluster on chromosome 12q. These studies are continuing.

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 the isotretinoin or etretinate. Most of these conditions have no effective alternative therapy. We studied the pharmacokinetics of etretinate absorption, demonstrating that significantly higher blood levels occur after administration with a fat load (milk). Because of these studies, the drug is routinely administered with meals.

A major toxicity limiting the use of systemic retinoids is their teratogenicity. Some retinoids, such as etretinate are fat stored. For these drugs the teratogenic risk can persist after the discontinuation of treatment. Our study of the pharmacokinetics of etretinate elimination detected blood levels of the drug up to 3 years after therapy was discontinued, far longer than previously known. We established that the half-life of elimination varies greatly between patients, may be as long as 6 months, and that the elimination of this drug is slower in patients with greater amounts of body fat. Clarifying the pharmacokinetics of these teratogenic drugs is crucial to their safe use in females of childbearing potential.

Chronic retinoid bone toxicity has been extensively studied in these patients. This toxicity is similar to the disorder DISH (diffuse idiopathic skeletal hyperostosis). Our group was the first to identify the high frequency of involvement of peripheral skeletal tissue involvement in etretinate treated patients. This peripheral skeletal toxicity also occurs with isotretinoin. Monitoring of these patients will enable us to further define the parameters of these toxicities.

Osteoporosis has been observed in chronic hypervitaminosis A. Our unique ability for long term follow-up of these patients allows us to assess this potential toxicity in patients chronically treated with isotretinoin or

etretinate. Dual photon bone densitometry is being performed on these patients to assess bone density.

Six patients with psoriasis were treated for eight weeks with intranasal Peptide T. Peptide T, which interacts with the CD4 receptor, is being studied in NIMH for the treatment of HIV associated dementia. The baseline neuropsychiatric and pharmacokinetic data obtained in these studies on a non-HIV infected population are being utilized to characterize the drug's actions in HIV patients. Only minimal benefit was observed using the intranasal preparation. A follow-up double-blind, study utilizing a topical preparation applied directly to psoriatic lesions is underway. Time devoted to these AIDS-related activities is 5%.

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 continuing 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. A collaborative study with Allen Bale, M.D. to map the locus of the gene for the nevoid basal cell carcinoma (Gorlin's) syndrome has identified an area of consistent allelic loss of chromosome 9q31 in the tumors from sporadic basal cell carcinomas and hereditary tumors. This suggests that the cause of the tumors in this condition may be the of loss of function of a tumor suppressor gene. Linkage analysis demonstrated tight linkage between the Gorlin syndrome gene and a genetic marker in this region. This study is continuing to further characterize the gene.

We have also formed a collaborative group with Sherri Bale and Peter Steinert designed to better clinically characterize and to map the genes for the disorders of keratinization. We have begun to identify and characterize clinical heterogeneity between families with epidermolytic hyperkeratosis. In addition, we have successfully identified linkage of the epidermolytic

hyperkeratosis gene to the type II keratin gene cluster on chromosome 12q. Further study of this gene and the other disorders of keratinization are ongoning.

Publications:

Gailani MR, Bale SJ, Leffell DJ, DiGiovanna JJ, Peck GL, Poliak S, Drum MA, Pastakia B, McBride OW, Kase R, Greene M, Mulvihill JJ, Bale AE. Developmental defects in Gorlin syndrome related to a putative tumor suppressor gene on chromosome 9, Cell 1992;69:111-117.

O'Connell PG, Gerber LH, DiGiovanna JJ, Peck GL. Arthritis in patients with psoriasis treated with gamma-interferon, J Rheumatol 1992;19:80-2.

Kraemer KH, DiGiovanna JJ, Peck GL. Chemoprevention of skin cancer in xeroderma pigmentosum, J Derm (in press).

Compton JG, DiGiovanna JJ, Santucci SK, Kearns KS, Amos CI, Abangan DL, Korge BP, McBride WO, Steinert PM, Bale SJ. Linkage of epidermolytic hyperkeratosis to the type II keratin gene cluster on chromosome 12q, Nature Genetics (in press).

DiGiovanna JJ. Diversity of melanoma in humans, Clin Chem Enzym Comm (in press).

DiGiovanna JJ, Kraemer KH, Peck GL, Abangan DL. Oral isotretinoin is effective in the prevention of skin cancer in patients with xeroderma pigmentosum. In: Louisiana State University Press. Vitamins and cancer prevention (in press).

Peck GL, DiGiovanna JJ, Kraemer KH, Tangrea JA, Gross EG. Treatment and prevention of basal cell carcinoma with oral isotretinoin. In: DePalo G, ed. Progress and perspectives in chemoprevention of cancer, Raven Press (in press).

DiGiovanna JJ. Prevention of cancer of the skin due to sun exposure. In: DeVita VT, Hellman S, Rosenberg SA, eds. Philadelphia: J.B. Lippincott Co., (in press).

Peck GL, DiGiovanna JJ. Retinoids. In: Fitzpatrick TB, et al eds. Dermatology in general medicine. New York: McGraw-Hill, 1992 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 CB 03667-08 D

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of Epidermal Cell Adhesion Molecules with Autoantibodies

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

P.I.: John R. Stanley, M.D., Medical Officer, Dermatology Branch, DCBDC, NCI
OTHER: Masayuki Amagai, M.D., Visiting Fellow, Dermatology Branch, DCBDC, NCI
George Elgart, M.D., Medical Staff Fellow, Dermatology Br., DCBDC, NCI
Sarolta Karpati, M.D., Visiting Scientist, Dermatology Br., DCBDC, NCI
Ronald Prussick, M.D., Medical Staff Fellow, Dermatology Br., DCBDC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 6	PROFESSIONAL: 5	OTHER: 1

CHECK APPROPRIATE BOX(ES)

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

B

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

The general and long-term goal of my laboratory is to study autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis. Specifically, we have found that autoantibodies from these patients, who develop blistering diseases due to defects in epidermal cell adhesion, are directed against adhesion molecules. We are characterizing, by immunochemical and molecular biologic means, the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). This then allows us to study their cell biologic function. BP antigen is a component of the hemidesmosome, a basal-cell substrate adhesion junction. We have cloned cDNA with the full length coding sequence for this molecule. Analysis of its deduced amino acid sequence indicates that BP antigen has marked amino acid and structural homology with desmoplakin I, a desmosome plaque protein, and with plectin, a keratin-associated protein. We have also determined that there is one gene encoding BP antigen and that it is grossly normal in patients with junctional epidermolysis bullosa, a hereditary disease with abnormal hemidesmosomes. We have also cloned cDNA with the full length coding sequence for PV antigen. The deduced amino acid sequence of this antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules and is closely related to the PF antigen. PV patients have antibodies against the amino-terminal domain of this molecule, an area thought to be important in its adhesion function, and these antibodies can cause loss of adhesion of epidermal cells in an animal model of disease.

PHS 6040 (Rev 1/84) U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

Project Description

Major Findings:

Overlapping cDNAs encoding the entire 230-kD bullous pemphigoid (BP) antigen have been cloned by screening cDNA libraries and by using the polymerase chain reaction rapid amplification of cDNA ends (RACE) technique.

Sequence analysis indicates that BP antigen is similar in structure, charge periodicity, and amino acid sequence to desmoplakin I, a desmosomal plaque protein, and to plectin, a keratin-binding protein.

There is a single human gene for the 230-kD BP antigen. A related gene exists in mammals, but not birds or lower vertebrates.

There are no gross abnormalities (e.g. insertions, deletions) of the BP antigen gene in junctional epidermolysis bullosa patients, even though they are known to have a defect in hemidesmosomes.

cDNA cloning of pemphigus vulgaris (PV) antigen indicates that it is in the cadherin family of calcium-dependent cell adhesion molecules, but it is a unique cadherin, limited in distribution to stratified squamous epithelia.

PV antigen is most closely related to desmoglein I, which is also a cadherin and is the pemphigus foliaceus antigen.

PV sera bind immunodominant epitopes in the amino-terminal extracellular domain of PV antigen, a region of cadherins thought to be important for their homophilic binding function.

Antibodies from PV sera that bind the amino-terminal extracellular domain of PV antigen are capable of causing the pathology of PV blisters (i.e. loss of epidermal cell adhesion) in a neonatal mouse model of disease.

Publications:

Amagai M, Elgart G, Klaus-Kovtun V, Stanley JR. Southern analysis of the 230-kD bullous pemphigoid antigen gene in normal humans, animals, and patients with junctional epidermolysis bullosa, J Invest Dermatol 1991;97:249-253.

Tanaka T, Parry DAD, Klaus-Kovtun V, Steinert P, Stanley JR. Comparison of molecularly cloned bullous pemphigoid antigen to desmoplakin I confirms that they define a new family of cell adhesion junction plaque proteins, J Biol Chem 1991;266:12555-12559.

Stanley JR. Pemphigus. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF, eds. Dermatology in General Medicine, McGraw-Hill (in press).

Stanley JR. Bullous pemphigoid, cicatricial pemphigoid and chronic bullous disease of childhood. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF, eds. Dermatology in General Medicine, McGraw-Hill (in press).

Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion, Cell 1991;67:869-877.

Green KJ, Virata MLA, Elgart GW, Stanley JR, Parry DAD. A comparative structureal analysis of desmoplakin, bullous pemphigoid antigen, and plectin: members of a new gene family involved in organization of intermediate filaments, Int J Biol Macromol (in press).

Amagai M, Karpati S, Prussick R, Klaus-Kovtun V, Stanley JR. Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic, J Clin Invest (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 03669-03 D

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Regulation of Cutaneous Accessory Cell Activity in Health and Disease

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

P.I.: Mark C. Udey, M.D., Ph.D., Expert, Dermatology Branch, DCBDC, NCI

Other: Teresa Borkowski, M.D., Medical Staff Fellow, DB, DCBDC,, NCI
Aimin Tang, M.D., Visiting Fellow, DB, DCBDC, NCI
Min-Geol Lee, M.D., Ph.D., Visiting Fellow, DB, DCBDC, NCI

COOPERATING UNITS *(if any)*

Len Neckers, Ph.D., Senior Investigator, CPB, DCT, NCI

LAB/BRANCH: Dermatology Branch

SECTION:

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

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
4.92	3.92	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The goal of the laboratory is to understand the cell biology of epidermal Langerhans cells (LC); especially those aspects that explain special properties of LC such as their susceptibility to functional inactivation by ultraviolet (UV) radiation, their ability to initiate primary immune responses and their propensity to localize in the epidermis. We have demonstrated that doses of UVB that functionally inactivate LC prevent enhanced expression of ICAM-1 (CD54), and that interaction of ICAM-1 on LC with LFA-1 on T cells is prerequisite for LC-T cell cluster formation and anti-CD3 mAb-induced T cell proliferation. We have also observed that cultured LC (that express high levels of ICAM-1 and perhaps other adhesion molecules) are resistant to doses of UVB that inactivate fresh LC. These studies are confounded by subsequent studies that showed that doses of UVB radiation that modulate ICAM-1 expression and LC accessory cell activity were ultimately cytotoxic for LC. Similar cytotoxic effects of UV for LC were noted with biologically active amounts of UVC and psoralen + UVA radiation in vitro, and may be responsible for certain immunosuppressive effects of low dose UV radiation in vivo. Studies designed to identify additional important adhesion (or costimulatory) molecules on LC are ongoing. mRNA encoding the murine homologue of the B cell activation antigen B7 (BB1), a costimulatory molecule thought to be important in the activation of Th1 cells, has been reverse transcribed, amplified using the polymerase chain reaction and detected in samples of epidermal cell RNA by autoradiography after fluid-phase hybridization with complementary radiolabelled synthetic oligonucleotides. Quantitative studies have revealed that cultured LC express 100-1000x more B7 mRNA than fresh LC or keratinocytes (KC) and 10-100x more B7 mRNA than the reference cell line CH-1. Cultured murine LC also provide costimulatory activity for human T cells in a xenogeneic assay that we believe measures B7 functional activity. Expression of B7 by cultured LC may be largely responsible for the ability of LC to activate unprimed T cells. Very recent studies may also provide insight into mechanisms that promote the localization of LC in epidermis. We have determined that LC express cadherins, that LC adhere to E-cadherin expressing cells in vitro, and that anti-E-cadherin mAb abrogates LC-KC binding. E-cadherin expression by LC reflects endogenous synthesis since LC also contain E-cadherin mRNA. The role that cadherins play in LC biology and in the localization of other leukocytes in epithelia is under investigation.

Project Description

Major Findings

In the past several years, most effort in the laboratory has been directed towards increasing our understanding of local (cutaneous) immunosuppressive effects of ultraviolet (UV) B radiation. We have focused on effects of UVB on epidermal Langerhans cells (LC) because we believe that adverse effects of low dose UVB radiation on LC may explain these immunosuppressive phenomena. We observed that doses of UVB radiation that inhibited the ability of LC to support the proliferative response of unprimed murine T cells to anti-CD3 mAb also prevented cluster formation between LC and T cells and selectively inhibited enhanced ICAM-1 (CD54) expression by LC in vitro. Because anti-ICAM-1 mAb inhibited both cluster formation and anti-CD3 mAb-induced T cell proliferation, we reasoned that effects of UVB radiation might be directly mediated through effects of UVB radiation on ICAM-1 expression by LC. Consistent with this hypothesis, we subsequently found that 72 h cultured LC (that expressed high levels of ICAM-1) were resistant to doses of UVB radiation that completely inhibited the accessory cell function of freshly-isolated LC.

Surprisingly, cultured LC were functionally resistant to UVB radiation even though approximately two-thirds of the irradiated cells died (became permeable to propidium iodide) within 24 h after exposure to UVB radiation. The functional resistance of cultured LC to doses of UVB radiation that are cytotoxic can potentially be explained in several ways. First, we determined that T cells became committed to proliferate within 4-8 h after exposure to anti-CD3 mAb in the presence of cultured LC. In contrast, a 24-30 h co-culture period was required for irreversible T cell activation if T cells were exposed to anti-CD3 mAb in the presence of freshly-isolated LC. Second, nonviable (paraformaldehyde-fixed) cultured LC retain accessory cell activity, whereas fixed freshly-isolated LC do not support anti-CD3 mAb-induced T cell proliferation.

The potential cytotoxicity of low dose UVB radiation for LC in situ is controversial. In light of the pronounced cytotoxicity of low dose UVB radiation for cultured LC in vitro, we studied cytotoxic effects of UVB on freshly-isolated LC more systematically. Prior studies in our laboratory had demonstrated that UVB was not cytotoxic acutely and did not adversely effect recovery of LC from culture 24 h after UVB exposure. In addition, exposure of fresh LC to UVB radiation selectively blunted enhanced ICAM-1 expression by LC in vitro after 24 h, without affecting class II MHC antigen, CD45 antigen, $Fc\gamma$ receptor and CD11b/CD18 (Mac-1) expression. Further studies revealed that although similar numbers of LC were recovered from cultures of UVB-irradiated and unirradiated cells, recovery of LC from cultures of irradiated cells was 50 and 10 % of control 48 and 72 h after UVB exposure. Effects of UVB on LC recovery were not reversed by TNFα or GM-CSF + IL-1α (keratinocyte-derived cytokines known to support LC survival in vitro), and reflected cytotoxicity of UVB for LC and not selective modulation of class II MHC antigen expression as previously suggested by others. Additional experiments with UVC and psoralen + UVA (PUVA) radiation also demonstrated that amounts of UVC or PUVA that inhibited LC accessory cell function were ultimately cytotoxic for LC in vitro.

We presume that both immunomodulatory and cytotoxic effects of UV on LC are mediated through UV-induced covalent modifications of DNA that adversely effect transcription of the ICAM-1 gene, and housekeeping genes as well. Initial attempts to determine if UVB inhibited transcription of the ICAM-1 gene in LC (utilizing the polymerase chain reaction in tandem with fluid phase hybridization to quantitate ICAM-1 mRNA levels in LC) were unsuccessful because ICAM-1 mRNA is relatively abundant in keratinocytes and it is difficult to rid LC preparations of contaminating keratinocytes. These studies may be continued if it becomes possible to separate LC from contaminating keratinocytes routinely. Because the potential cytotoxicity of UV for LC (although it may explain some or all of the local immunosuppressive effects of UV radiation in vivo) confounds the interpretation of functional studies, additional studies of effects of UV on LC in vitro are not anticipated.

We have proposed that cultured LC exhibit potent accessory cell activity and are functionally resistant to low dose UV radiation and chemical fixation because they express adhesion (or costimulatory) molecules that enable them to activate unprimed T cells. Freshly-isolated LC (that are deficient in these cell surface molecules) are correspondingly less effective accessory cells. We are actively involved in defining the array of costimulatory molecules expressed by cultured LC utilizing immunochemical, biochemical and molecular biologic techniques. Candidate costimulatory molecules include several members of the immunoglobulin gene superfamily; ICAM-1 (CD54), LFA-3 (CD58), the B cell activation antigen B7/BB1 and VCAM-1. We have previously reported that cultured LC express high levels of ICAM-1, and that ICAM-1 appears to be critically involved in physical interactions between LC and T cells.

Studies of B7 expression by murine LC are in progress. Because appropriate immunochemical reagents are not available, we have taken a molecular approach. We have reverse transcribed B7 mRNA, amplified cDNAs utilizing the polymerase chain reaction (PCR) and B7 specific primers, and detected PCR products after fluid phase hybridization with radiolabelled complementary oligonucleotide probes and radioautography. By titrating primer concentrations, cellular RNA concentrations and PCR cycle numbers this system can be made semi-quantitative (such that signal strength in an autoradiograph is proportional to RNA added). We have determined that cultured LC express very high levels of B7 mRNA (100-1000x) relative to fresh LC or keratinocytes, and also express 10-100x higher levels than those of a reference cell line (CH-1). Utilizing a xenogeneic costimulation assay, we have also demonstrated that cultured LC exhibit costimulatory activity that probably reflects B7 expression. Studies that will prove that the costimulatory activity measured in this xenogeneic assay system is B7 are in progress.

Expression of LFA-3 will be studied utilizing a murine CD2-human IgM fusion protein (provided by Dr. Klaus Karjalainen) and VCAM-1 can be assessed utilizing a monoclonal Ab recently described by Dr. Paul Kincade). Having defined the array of costimulatory molecules expressed by cultured LC, we will attempt to manipulate expression of the various costimulatory molecules using antisense oligonucleotides provided by Dr. Len Neckers (CPB, DCT, NCI). Pilot studies will be carried out with ICAM-1 antisense oligonucleotides, and a battery of cultured LC expressing differing arrays of adhesion molecules will be generated if feasible. We plan to study the functional importance of individual types of adhesion molecules on bona fide accessory cells using this collection of accessory cells as a source of costimulatory activites for various T cell subpopulations.

We have also recently become interested in identifying adhesion molecules that mediate adhesion between leukocytes and keratinocytes, and have made what we believe is a significant observation. We have demonstrated that LC express several members of the cadherin gene superfamily and that E-cadherin mediates adhesion of LC to keratinocytes in vitro. Correspondingly, it seems likely that cadherins may play a role in the localization of LC in epidermis in vivo. Our results are of additional interest because cadherins have not previously been identified on leukocytes and have not previously been implicated in leukocyte trafficking. We are actively studying the role that cadherins play in LC biology and are attempting to generalize these observations by examining other epithelial leukocytes for cadherin expression.

Publications:

Journal Articles:

Udey MC, Peck RD, Pentland AP, Schreiner GF, Lefkowith JB. Antigen presenting cells in essential acid deficient murine epidermis: Keratinocytes bearing class II MHC (Ia) antigens may potentiate the accessory cell function of Langerhans cells, J. Invest. Dermatol. 1991;96: 950-958.

Tang A, Udey MC. Differential sensitivity of freshly isolated and cultured murine Langerhans cells to ultraviolet B radiation and chemical fixation, Eur. J. Immunol. 1992;22: 581-586.

Tang A, Udey MC. Effects of UV radiation on murine epidermal Langerhans cells: Doses of ultraviolet radiation which modulate ICAM-1 (CD54) expression and inhibit Langerhans cell function cause delayed cytotoxicity in vitro, J. Invest. Dermatol. (in press).

Journal Supplements:

Tang A and Udey MC. Doses of ultraviolet radiation that modulate accessory cell activity and ICAM-1 expression are ultimately cytotoxic for murine epidermal Langerhans cells, J. Invest. Dermatol. (in press).

Chapters in Books:

Udey MC. Atopic and contact dermatitis. In: Kelley WN, ed. Textbook of internal medicine. JB Lippincott, 1992;985-987.

Udey MC and Goslen JB. Allergic urticaria and erythema multiforme. In: Kelley WN, ed. Textbook of internal medicine. JB Lippincott, 1992;987-989.

Udey MC and Goslen JB. Cutaneous reactions to drugs. In: Kelley WN, ed. Textbook of internal medicine. JB Lippincott, 1992;989-990.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1991 through September 30, 1992

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area a broad range of immunological investigations are carried out in patients with primary and acquired immunodeficiency diseases that are associated with a high incidence of neoplasia, as well as in patients with malignancy, especially T- and B-cell leukemias. These studies focus on the definition of disorders in the control of the human immune response that underlie malignant and immunodeficiency diseases. Furthermore, they are directed toward developing rational approaches for the prevention and treatment of cancer, primary immunodeficiency diseases and AIDS. These studies include: 1) The characterization of transacting regulatory factors that mediate lymphocyte-specific gene transcription. The scientific focus of this area is the purification of the transactivating factors, the cloning of the genes encoding these factors and the definition of their mode of action at a molecular level. 2) Somatic gene therapy for human genetic immunodeficiency diseases. 3) Genetic control of the immune response. One emphasis of this area is the development of a novel method for predicting molecular structures recognized by T cells and the applications of this algorithm to the development of vaccines aimed at preventing and treating AIDS and cancer. 4) Identification, purification, and molecular genetic analysis of the multichain interleukin-2 receptor on normal and malignant lymphocytes. A major emphasis is placed on the development of different forms of IL-2 receptor-directed therapy. 5) Analysis of action of immunoregulatory cells including helper T cells, suppressor T cells, and macrophages that regulate antibody responses, and on studies of disorders of immunoregulatory cell interactions, in immunodeficiency diseases, in individuals with leukemias of these immunoregulatory cells. 6) Isolation and characterization of novel lymphokines, biological response modifiers and oligosaccharides that regulate the human immune response.

The second major goal of the Metabolism Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic diseases as well as those with 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 normal growth factors, especially insulin-like growth factors that participate in the hormonal control of normal and malignant growth.

MOLECULAR ANALYSIS OF TRANSACTING FACTORS THAT MEDIATE GENE EXPRESSION

Dr. Lou Staudt's laboratory is taking three molecular approaches to understanding the development and function of lymphocytes. The first approach focuses on changes in the expression of transcription factors which take place when hematopoietic stem cells differentiate along the B cell lineage. In particular, Dr. Staudt has studied a novel population of mouse bone marrow progenitor cells which can differentiate, under appropriate conditions, into either B cells or myeloid cells. A variety of transcription factors studied were expressed in both the progenitor and differentiated cells. By contrast, two transcription factors, Oct-2 and LEF-1, are present at low or undetectable levels in the

progenitor cells and are strongly induced upon differentiation to the lymphoid, but not the myeloid, lineage. The molecular cloning of the lymphoid-restricted transcription factor, Oct-2 by Dr. Staudt helped to define the POU-domain transcription factor multigene family. Oct-2 and LEF-1 may play a role in commitment of a stem cell to the lymphoid lineage. The second approach involves the molecular cloning of novel lymphoid-restricted genes using subtractive hybridization techniques. One such gene, Ly-GDI, encodes a protein bearing striking homology to a regulator of the ras-like G protein, rho, and may regulate events during lymphocyte activation. Another protein, JAW-1, has homology to the coiled-coil region of myosin and, surprisingly, resides in the endoplasmic reticulum. Both of these proteins reveal tissue-specific regulation of cellular processes that were previously thought to behave similarly in all cell types. The final approach involves the rapid and large-scale sequencing of cDNAs derived from subtracted and conventional cDNA libraries prepared from normal human lymphocytes or human lymphoid malignancies such as Burkitt's lymphoma and chronic lymphocytic leukemia.

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 10^9 autologous ADA gene-corrected T cells into a 4-year-old girl with ADA deficiency SCID. Retroviral vectors were used to insert a normal human ADA gene into this girl's polyclonal peripheral blood T-cells which had been stimulated in tissue culture with an anti-T-cell receptor monoclonal antibody and IL2. The gene-corrected T cells were expanded 100-1000 fold and then returned intravenously within 2 weeks to maintain a polyclonal repertoire. This girl and a second ADA deficient patient have been treated 10-12 times over the past two 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. Dr. Blaese's laboratory has also developed a unique new approach to direct gene therapy of cancer using instilation of murine fibroblasts producing retroviral vectors directly into tumors in situ. Using vectors for herpes simplex thymidine kinase, he has shown cure of brain tumors in rats following systemic administration of Ganciclovir. In addition Dr. Blaese has continued working on the development of succinylacetone as a clinically useful immunosuppressive compound. He showed that succinylacetone has profound dual system immunosuppressive effects in rats, mice, dogs, miniature swine and non-human primates. It is effective in preventing allograft rejection, graft vs host disease (GVHD), antibody production in these experimental animal models and has shown utility in treating several different autoimmune disorders including experimental autoimmune uveitis and adjuvant arthritis.

MECHANISMS OF ANTIGEN-PRESENTATION AND T-LYMPHOCYTE RECOGNITION: APPLICATION TO VACCINE DESIGN

Dr. Berzofsky's lab has studied the mechanisms by which T cells recognize antigens presented on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and the application of these principles to the design of synthetic vaccines for AIDS and cancer. They have characterized a peptide fragment of the HIV-1 envelope protein recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) using a new system in which CTL are stimulated by peptide bound to purified Class I MHC molecules attached to plastic microtiter wells. They have identified a 10-residue HIV peptide that is a million times more active than the original 15-residue peptide in the absence of serum, and shown that the longer peptide requires processing by angiotensin converting enzyme (ACE) in serum, and is inhibited by the ACE-inhibitor captopril. They also showed that the Class I molecule plays two roles in CTL activation, one to present the peptide and the other requiring only a conserved region on the alpha 3 domain, presumably to interact with CD8. This result showed

that the MHC molecule binding CD8 does not have to be the same one presenting peptide. They have characterized the fine specificity of CTL for this peptide, and shown that non-crossreactive CTL distinguish aliphatic from aromatic residues at a single position. This finding led to the discovery of a way to induce broadly crossreactive CTL against multiple variants of HIV by stimulation with a chimeric peptide. This peptide was also found to crossreact with a superficially unrelated peptide from the gp41 region of the envelope, even when presented by 4 different Class I MHC molecules. The crossreactive CTL use a limited repertoire of receptor variable regions, Vβ8 and Vβ14. This CTL site, which also is a target of neutralizing antibodies, has been coupled to sites identified by Berzofsky that stimulate T helper cells in mice and humans of multiple MHC types, and the resulting candidate synthetic vaccine has been found to induce extremely high titers of neutralizing antibodies in mice, as well as specific CTL killing of HIV-1 envelope expressing cells. A Phase I human immunotherapy trial with this construct is being planned. Also, an early diagnostic test is being developed. Dr. Berzofsky also showed that mice infected with schistosomiasis and then with a vaccinia virus recombinant expressing HIV envelope made reduced IL-2, interferon, and CTL responses specific for HIV envelope and had difficulty eliminating the virus. This may account in part for the rapid spread of AIDS in Africa, where such parasites are endemic. The same approach has been applied to cancer vaccines aimed at inducing CTL to mutant peptides corresponding to oncogene mutations (especially in p53 and ras, that are common in many human cancers), that could kill tumor cells. Dr. Berzofsky's lab has succeeded in inducing CTL to a peptide corresponding to a p53 tumor suppressor gene mutation, that will kill tumor targets expressing the mutant p53 gene. This demonstrates that such mutant oncogene products, although not expressed on the cell surface, can serve as targets of specific cancer immunotherapy. Also, a CTL determinant of hepatitis C virus has been found.

THE MULTICHAIN IL-2 RECEPTOR: MOLECULAR CHARACTERIZATION AND USE AS A TARGET FOR IMMUNOTHERAPY

Effective therapy of cancer using antibody mediated therapy has been elusive. A number of factors explain the low therapeutic efficacy observed. Unmodified monoclonal antibodies are immunogenic and elicit a human immune response to the murine antibody. Moreover, mouse monoclonal antibodies are not cytocidal against neoplastic cells in humans and in most cases are not directed against a vital cell surface structure such as a receptor for a growth factor required for tumor cell proliferation. Dr. Thomas Waldmann has addressed these issues by using the IL-2 receptor as a target for monoclonal antibody immunotherapy, by genetic engineering to create less immunogenic and more effective monoclonal antibodies, and by arming such antibodies with toxins or radionuclides to enhance their effector action. Dr. Waldmann previously identified two peptides that bind IL-2: the 55 KD protein IL2Rα chain reactive with the anti-Tac monoclonal antibody, and the 70/75 kD IL-2Rβ protein reactive with a monoclonal antibody termed Mikβ1. He proposed a multichain model for the high affinity receptor in which both IL-2Rα- and IL-2Rβ-binding proteins are associated in a receptor complex. Dr. Waldmann recognized the value of the IL-2 receptor as a therapeutic target. Normal resting T cells, B cells and monocytes do not express the IL-2 receptor. In contrast this receptor is expressed by the abnormal cells of patients with certain forms of leukemia, autoimmune disease, and those rejecting allografts.

Dr. Nelson and his colleagues identified a soluble form of the p55 component (Tac protein) of the human interleukin-2 receptor in the supernatants of activated T-cells, B-cells, and monocytes in vitro and in the serum and urine of normal individuals in vivo. Elevated levels of soluble IL-2Rα were found in the sera of patients with human retroviral diseases including the adult T-cell leukemia, hairy cell leukemia, and the acquired immunodeficiency syndrome. In adult T-cell leukemia and hairy cell leukemia patients the serum level of IL-2Rα was indicative of tumor burden and favorable responses

to therapy were associated with reductions in the serum level of Tac protein. Elevations of serum IL-2Rα were also indicative of allograft rejection episodes in patients with liver and heart-lung transplants. Dr. Nelson also demonstrated that patients with autoimmune diseases had elevated levels of IL-2Rα in serum and joint fluids. These studies have demonstrated that the measurement of soluble Tac protein in various body fluids is useful in monitoring certain neoplastic and immune-mediated events in vivo.

Dr. Waldmann designed a novel form of therapy, IL-2 receptor directed therapy, to exploit this difference in IL-2 receptor expression between normal resting cells and abnormal T cells that cause disease. Initially Dr. Waldmann focused his IL-2 receptor directed therapeutic studies on patients with adult T-cell leukemia (ATL). ATL is an aggressive disorder with no known curative chemotherapy that kills patients on average in 20 weeks. All populations of leukemic cells examined by Dr. Waldmann from patients with HTLV-I-associated ATL express very large numbers of IL-2 receptors identified by the anti-Tac monoclonal antibody. Dr. Waldmann initiated a therapeutic trial using unmodified anti-Tac monoclonal antibody in the treatment of patients with ATL with the goal of preventing the interaction of IL-2 with the IL-2 receptor thus depriving the malignant cells of a growth factor required for their proliferation and survival. The patients studied did not suffer any toxicity. Seven of the 20 patients studied underwent a remission; in three cases a complete remission lasting from 8 to over 27 months following initiation of anti-Tac therapy. Although use of such murine antibodies is of value in the therapy of human diseases, their effectiveness is limited by the fact that rodent monoclonal antibodies often induce a human immune response to them. To circumvent this difficulty genetically engineered antibody variants of anti-Tac were produced by combining the rodent genetic elements encoding the hypervariable regions with human, constant and framework region genes. Dr. Waldmann showed that the "humanized" version of the anti-Tac monoclonal antibody is dramatically less immunogenic than the parent mouse monoclonal. Furthermore, he showed that the "humanized" version of anti-Tac manifests a killing ability directed toward human tumor cells termed antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. With the lowered immunogenicity, improved pharmacokinetics, and a new effector function antibody-dependent cellular cytotoxicity it is hoped that there will be a substantial improvement in the therapeutic efficacy of this genetically engineered monoclonal antibody. Dr. Waldmann confirmed this predicted improved effectiveness in preclinical animal models and plans to initiate therapeutic trials with "humanized" anti-Tac in patients with IL-2 receptor expressing malignancies. A clinical trial with this antibody has been initiated in patients with IL-2 receptor-expressing leukemia and lymphoma and in individuals undergoing graft versus host disease. In parallel studies a "humanized" version of Mikß1 that blocks binding to the IL-2Rß component has been generated by combining the complementarity determining regions of MikB1 with human immunoglobulin framework and constant regions. As with anti-Tac "humanized" Mikß1 manifests antibody-dependent cellular cytotoxicity. Furthermore, the addition of "humanized" Mikß1 that blocks the interaction of IL-2 with the IL-2Rß subunit complements the anti-IL-2Rα chain antibody anti-Tac in inhibiting IL-2 induced proliferation.

Drs. Waldmann and David Nelson extended the clinical therapeutic implications of monoclonal antibodies by focusing on the use of these agents as carriers of cytotoxic agents. Here the goal is to maintain the specificity of the monoclonal antibody while increasing its capacity to kill unwanted cells by coupling toxins or radionuclides to it. They developed cytotoxic agents wherein α- and ß-emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. For example Dr. Waldmann showed that bismuth-212, an α-emitting radionuclide conjugated to anti-Tac was well-suited for a therapeutic role. In parallel studies Drs. Waldmann and Nelson bound the ß-emitting radionuclide Yttrium-90 to anti-Tac using chelates that neither damage the antibody nor permit the elution of radiolabeled Yttrium from it. Following efficacy and toxicity studies in animal models, they initiated

a dose escalation trial with Yttrium-labeled anti-Tac for the treatment of HTLV-I-associated adult T-cell leukemia (ATL). Eleven of the 15 patients underwent a partial or complete remission following Yttrium-90 anti-Tac therapy. Thus it is hoped that Yttrium-90 chelated to "humanized" Mikß1 will prove to be effective, relatively nontoxic agents for the treatment of an array of human leukemias.

ISOLATION AND CHARACTERIZATION OF BIOLOGICAL RESPONSE MODIFIERS AND OLIGOSACCHARIDES THAT REGULATE HUMAN IMMUNE RESPONSES

Dr. Andrew Muchmore's interest in the functional role of defined glycopeptides and oligosaccharides as regulatory signals in their own right has led to a clearer appreciation of the physiologic role of these structures in regulating the cell cycle and the state of cell activation as well as the view that these compounds represent a new class of agents with broad pharmaceutical activities. Dr. Bibhuti Mishra, working with Dr. Muchmore extended previous observations to implicate a common mechanism for the activity of these pluripotent glycopeptides. Their data demonstrate that high mannose glycopeptides in a structurally-specific fashion act directly in the nucleus to regulate gene transcription. Using the AP-1 model first described in HeLa cells, Dr. Muchmore showed that Mannose-5 phosphate blocks the ability of the heterodimeric transcription factors fos-jun to bind to their consensus DNA binding region. These observations have been expanded to show a functional effect not only on in vitro transcription but also on cellular proliferation. Specific mannose structures block the unbridled proliferation of HeLa cells and at least partially induce contact growth inhibition. This ability to apparently reverse the malignant phenotype of HeLa cells suggests that these studies offer a new avenue of research in efforts to understand and control malignant cell growth. In addition, studies with transfected fibroblast lines from patients show that disorder of CNS development in mannosidosis is probably caused by inhibition of AP-1-mediated gene transcription.

In related studies Dr. Muchmore has defined a high affinity receptor specific for uromodulin, an inhibitor of lymphocyte proliferation. Furthermore, he has isolated and partially sequenced a cDNA clone from a human testis library which represents the human homolog of a sea urchin sperm protein known to have homology to uromodulin and to be functionally important in acrosomal body formation.

INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS

Dr. Peter Nissley has been studying the mechanism of action of insulin-like growth factors (IGF-I and IGF-II), focusing on the function of the two receptors that bind these ligands, the IGF-II/mannose 6-phosphate receptor and the IGF-I receptor. Based on earlier observations from his laboratory that IGF-II inhibited the cellular uptake of the lysosomal enzyme, ß-galactosidase, by inhibiting binding to the IGF-II/M6P receptor, Dr. Nissley and his colleagues proposed that endogenous IGF-II might modulate the cellular trafficking of lysosomal enzymes by this receptor. To test this hypothesis Dr. Nissley's laboratory will attempt to transfect a human breast cancer cell line, MCF-7, with the IGF-II gene and examine the extracellular versus intracellular distribution of newly synthesized lysosomal enzymes in the transfected cells. In order to identify clones of transfected MCF-7 cells that are producing large quantities of IGF-II, a rapid and sensitive assay that can be performed directly on conditioned media is required. To this end, Dr. Nissley's laboratory has developed a dot-blot assay based on Amersham enhanced chemiluminescence (ECL) methodology. This chemiluminescence dot-blot method detects picogram quantities of IGF-II and is therefore 100 times more sensitive than conventional radioimmunoassays for IGF-II. An additional advantage of this assay is that it can be performed in only 5-6 hrs.

The IGF-I receptor is a member of a large family of growth factor receptors with intrinsic tyrosine

kinase activity. Unlike several other members of this family such as the EGF and PDGF receptors, relatively little is known about the early postreceptor events in IGF-I receptor signaling. A number of growth factors and cytokines have been shown to stimulate the hydrolysis of plasma membrane phosphatidylinositol, generating the second messengers, diacylglycerol (DAG) and inositol phosphates. Dr. Nissley asked whether the IGF-I receptor utilizes this pathway in the human osteosarcoma cell line, MG63, by measuring DAG levels following addition of IGF-I to growth-arrested cells. There was no increase in DAG following addition of IGF-I, arguing against utilization of the phosphatidylinositol pathway in signaling by the IGF-I receptor.

In 1991, Roback and her colleagues reported the loss of one copy of the IGF-I receptor gene in a patient with deletion of the distal long arm of chromosome 15, and proposed that the severe growth retardation seen in the 15q deletion syndrome is related to the reduced gene dosage of the IGF-I receptor. Dr. Nissley has initiated experiments to test this proposal by studying IGF-I receptor expression and function in fibroblasts from two patients with the 15q deletion syndrome. Using quantitative Southern blotting, Dr. Nissley's laboratory showed that the level of the IGF-I receptor gene was reduced by approximately 50% in the patients' fibroblasts compared to controls. In addition, the cell surface expression of the IGF-I receptor as measured by binding of radiolabeled IGF-I, was also significantly reduced. To test the function of the IGF-I receptor in the fibroblasts, the stimulation of uptake of the amino acid analog, N-methyl-[14C]a-aminoisobutyric acid by IGF-I was examined. There was no significant difference between the patients' fibroblasts and control fibroblasts in ED50 for IGF-I, fold-stimulation over basal, or net uptake over time. Dr. Nissley concluded that although there is evidence for decreased expression of the IGF-I receptor protein in patients with only one gene copy of the IGF-I receptor, the function of the receptor as measured by the stimulation of amino acid transport, is not impaired.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CD-04002-23 MET

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunctions

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

PI:	Thomas A. Waldmann, M.D.	Branch Chief	MET, NCI
	Claude Kasten-Sportes, M.D.	Medical Staff Fellow	MET, NCI
	Erich Roessler, M.D.	Medical Staff Fellow	MET, NCI
	Jack Burton, M.D.	Biotechnology Fellow	MET, NCI
	Angus Grant, Ph.D.	Staff Fellow	MET, NCI
	Carolyn K. Goldman	Microbiologist	MET, NCI

(See next page)

COOPERATING UNITS *(if any)*

Laboratory of Molecular Biology, NCI
Radiation Oncology Branch, NCI

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12	10	2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

"B" 100%

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

Dr. Waldmann developed IL-2 receptor directed therapy for patients with leukemia. The scientific basis for this approach is provided by his observation that resting T cells do not express IL-2 receptors but receptors are expressed by the abnormal T cells of patients with lymphoma/leukemia, those with select forms of autoimmune disease, and individuals rejecting allografts. Dr. Waldmann proposed a multichain model for the high affinity IL-2 receptor involving two IL-2 binding proteins: a 55 kD (IL-2Rα) and a 75 kD (IL-2Rß) protein. To exploit the difference in IL-2 receptor expression between normal and malignant cells, he has initiated IL-2 receptor directed therapy in patients with human lymphotropic virus I (HTLV-I), associated adult T-cell leukemia (ATL). Using unmodified anti-Tac monoclonal antibody that reacts with IL-2Rα, one-third of the patients with ATL treated have undergone a remission. There was no toxicity observed, however, unmodified monoclonal antibodies are limited by their immunogenicity and their poor effector functions. To address these issues "humanized" anti-Tac was produced that retains the complementarity-determining regions from the mouse with the remainder of the molecule derived from human IgG1. This antibody is dramatically less immunogenic than the murine version, and, in contrast to the parent antibody, manifests antibody-dependent cellular cytotoxicity. A clinical trial with this antibody has been initiated in patients with IL-2 receptor-expressing leukemias and lymphomas as well as individuals with corticosteroid-resistant graft versus host disease. To enhance its effector function anti-Tac was armed with toxins and α- and ß-emitting radionuclides. In a clinical trial of ^{90}Y-anti-Tac in ATL 10 of the 15 patients with ATL underwent a partial or complete remission. Thus, the clinical application of IL-2 receptor directed therapy represents a new perspective for the treatment of certain neoplastic diseases, autoimmune disorders and for the prevention of allograft rejection.

Continuation of Professional Personnel for PHS 6040

Frank Hartmann, M.D.	Fogarty Visiting Associate	MET, NCI
Christian Peters, M.D.	Fogarty Visiting Associate	MET, NCI
Sikiru A. Tinubu, M.D.	Fogarty Visiting Associate	MET, NCI
Sara L. Zaknoen, M.D.	Medical Staff Fellow	MET, NCI

Project Description

Major Findings:

Effective therapy of cancer using unmodified monoclonal antibody mediated therapy has been elusive. A number of factors explain the low therapeutic efficacy observed. Unmodified monoclonal antibodies are immunogenic and elicit a human immune response to the murine antibody. Moreover, mouse monoclonal antibodies are not cytocidal against neoplastic cells in humans and in most cases are not directed against a vital cell surface structure such as a receptor for a growth factor required for tumor cell proliferation. Dr. Waldmann has addressed these issues by using the IL-2 receptor as a target for monoclonal antibody immunotherapy, by genetic engineering to create less immunogenic and more effective monoclonal antibodies, and by arming such antibodies with toxins or radionuclides to enhance their effector action. Dr. Waldmann previously identified two peptides that bind IL-2: the 55 KD protein IL2Rα reactive with the anti-Tac monoclonal antibody, and the 70/75 kD IL-2Rβ protein reactive with a monoclonal antibody termed Mikβ1. He proposed a multichain model for the high affinity receptor in which both IL-2Rα- and IL-2Rβ-binding proteins are associated in a receptor complex. Dr. Waldmann recognized the value of the IL-2 receptor as a therapeutic target. Normal resting T cells, B cells and monocytes do not express the IL-2 receptor. In contrast, this receptor is expressed by the abnormal cells of patients with certain forms of cancer or autoimmune disease and those rejecting allografts. Dr. Waldmann designed a novel form of therapy, IL-2 receptor directed therapy, to exploit this difference in IL-2 receptor expression between normal resting cells and abnormal T cells that cause disease. Initially, Dr. Waldmann focused his IL-2 receptor directed therapeutic studies on patients with adult T-cell leukemia (ATL). ATL is an aggressive disorder with no known curative chemotherapy that kills patients on average in 20 weeks. All populations of leukemic cells examined by Dr. Waldmann from patients with HTLV-I-associated ATL express very large numbers of IL-2 receptors identified by the anti-Tac monoclonal antibody. Dr. Waldmann initiated a trial using unmodified anti-Tac monoclonal antibody for the treatment of patients with ATL with the goal of preventing the interaction of IL-2 with the IL-2 receptor thus depriving the malignant cells of a growth factor required for their proliferation and survival. The patients studied did not suffer any toxicity. Seven of the 20 patients studied underwent a remission; in three cases a complete remission lasting from 8 to over 27 months following initiation of anti-Tac therapy. Patients who do not respond to anti-Tac still express the interleukin-2 receptor but do not produce IL-2 nor require this lymphokine for proliferation. Dr. Waldmann has identified several alterations in the leukemic cells of such patients including mutations in the IL-2Rβ subunit, a change in the IL-2R-associated src-type tyrosine kinase from lck to lyn and the production of a previously undefined lymphokine. Although murine antibodies are of value, their effectiveness is limited by the fact that rodent monoclonal antibodies often induce a human immune response to them. To circumvent this difficulty genetically engineered antibody variants of anti-Tac were produced by combining the rodent genetic elements encoding the hypervariable regions with human, constant and framework region genes. Dr. Waldmann showed that the "humanized" version of the anti-Tac monoclonal antibody is dramatically less immunogenic than the parent mouse monoclonal. Furthermore, he showed that the "humanized" version of anti-Tac manifests a killing ability directed toward human tumor cells termed antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. With the lowered immunogenicity, improved pharmacokinetics, and a new effector function antibody-dependent cellular

cytotoxicity, it is hoped that there will be a substantial improvement in the therapeutic efficacy of this genetically engineered monoclonal antibody. Dr. Waldmann confirmed this predicted improved effectiveness in preclinical animal models and has initiated therapeutic trials with "humanized" anti-Tac in patients with IL-2 receptor expressing malignancies. In parallel studies a "humanized" version of Mikß1 that blocks binding to the IL-2Rß component has been generated by combining the complementarity determining regions of MikB1 with human immunoglobulin framework and constant regions. As with anti-Tac "humanized" Mikß1 manifests antibody-dependent cellular cytotoxicity. Furthermore, "humanized" Mikß1 that blocks the interaction of IL-2 with the IL-2Rß subunit complements the anti-IL-2Rα chain antibody anti-Tac in inhibiting IL-2 induced proliferation.

Dr. Waldmann extended the clinical therapeutic implications of monoclonal antibodies by focusing on the use of these agents as carriers of cytotoxic agents. Here the goal is to maintain the specificity of the monoclonal antibody while increasing its capacity to kill unwanted cells by coupling toxins or radionuclides to it. He developed alternative cytotoxic agents wherein α- and ß-emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. For example he showed that bismuth-212, an α-emitting radionuclide conjugated to anti-Tac was well-suited for a therapeutic role. In parallel studies he bound the ß-emitting radionuclide yttrium-90 to anti-Tac using chelates that neither damage the antibody nor permit the elution of radiolabeled yttrium from it. Following efficacy and toxicity studies in animal models, he initiated a dose escalation trial with yttrium-labeled anti-Tac for the treatment of HTLV-I-associated adult T-cell leukemia (ATL). Ten of 15 patients with ATL studied underwent a partial or complete remission. It is hoped that Yttrium-90 chelated to "humanized" anti-Tac and to "humanized" Mikβ1 will prove to be effective, relatively nontoxic agents for the treatment of an array of human leukemias. Thus the new insights concerning the IL-2/IL-2 receptor system are providing a novel perspective for the treatment of certain neoplastic diseases, autoimmune disorders and for the prevention of allograft rejection.

Honors and Awards:

1991 The 19th Stanhope Bayne-Jones memorial Lectureship, Johns Hopkins University School of Medicine
1992 15th Bristol-Myers, Squibb Award for Distinguished Achievement in Cancer Research
1992 Honor Lecture, Oklahoma Medical Research Foundation and the Noble Foundation
1992 Stohlman Memorial Lecture - Wilsede Meeting on Modern Trends in Human Leukemia X
1992 Byrd Steuart Leavell Memorial Lecture, The Virginia Society of Hematology and Oncology
1992 Elected to Institute of Medicine of the National Academy of Sciences

Publications:

Grant AJ, Roessler E, Ju G, Tsudo M, Sugamura K, Waldmann TA. The interleukin 2 receptor (IL-2R): the IL-2R α subunit alters the function of the IL-2R β subunit to enhance IL-2 binding and signaling by mechanisms that do not require binding of IL-2 to IL-2R α subunit. Proc Natl Acad Sci USA 1992;89:2165-69.

Hakimi J, Chizzonite R, Luke DR, Familletti PC, Bailon P, Kondas JA, Pilson RS, Lin P, Weber DV, Mondini LJ, Tsien W-H, Levin JL, Gallati VH, Korn L, Waldmann TA, Queen C, Benjamin WR. Reduced immunogenicity and improved pharmacokinetics of humanized anti-Tac in cynomolgus monkeys. J Immunol 1991;147:1352-59.

Parenteau GL, Dirbas FM, Garmestani K, Brechbiel MW, Bukowski MA, Goldman CK, Souza LM, Clark R, Gansow OA, Waldmann TA. Yttrium-90 labeled anti-Tac in conjunction with granulocyte colony stimulating factor prolongs graft survival in primate allograft transplantation. In press.

Sanford KK, Parshad R, Price FM, Jones GM, Tarone RE, Eierman L, Hale P, Waldmann TA. Enhanced chromatid damage in blood lymphocytes after G_2 phase X-irradiation, a marker of the ataxia-telangiectasia gene. J Natl Cancer Inst 1990;83:1050-54.

Talmage DW, Bice DE, Bloom AC, Koller LD, Lamm ME, Luster MI, Meggs WJ, Munson AE, Rodgers KE, Rose NR, Schulte PA, Travis CC, Tucker ES, Vogt RF, Waldmann TA. Biologic Markers in immunotoxicology. Nat'l research Council, Washington DC: Nat'l Academy Press 1992;1-206.

Tendler CL, Greenberg SJ, Burton JD, Danielpour D, Kim S-J, Blattner WA, Manns A, Waldmann TA. Cytokine induction in HTLV-I associated myelopathy and adult T-cell leukemia: alternate molecular mechanisms underlying retroviral pathogenesis. J Cell Biochem 1991;46:302-1

Waldmann TA. Adult T-cell leukemia: prospects for immunotherapy. In: Gallo RC, Jay G, eds. The Human Retroviruses. San Diego: Academic 1991;319-34.

Waldmann TA. IL-2 and its receptor. In: Lechmann PF, Rosen F, Peters DK, eds. Clinical Aspects of Immunology, 5th Ed, in press.

Waldmann TA. Lymphokine receptor-directed therapy: a model for immune intervention in leukemia, autoimmunity and immunodeficiency. Clin Immunol and Immunopathol 1991;61:S37-S46.

Waldmann TA. The interleukin-2 receptor: a target for immunotherapy for leukemia and lymphoma. In: Origins of Human Cancer: a comprehensive review. Cold Spring Harbor Laboratory Press 1991;855-63.1991;855-63.

Waldmann TA. Interleukin-2 receptor directed therapy in autoimmune disease. In: Kahn C, ed. Early decisions in DMARD development II: biologic agents in autoimmune disease. Atlanta: Arthritis Foundation Press 1991;34-39.

Waldmann TA. IMMUNE RECEPTORS: targets for therapy of leukemia/lymphoma, autoimmune diseases and for the prevention of allograft rejection. Ann Rev Immunol 1992;10:675-704.

Waldmann TA (moderator). The IL-2 receptor as a target for immunotherapy in patients with Tac expressing leukemia, patients with autoimmune disorders, and individuals receiving organ allografts. Conference of the Combined Staff, NIH. Ann Intern Med 1992;116:148-60.

Waldmann TA. Clinical immunology: past, present and future challenges and prospects. Liber Amicorum, Utrecht, Neth J Med 1991;39:322-28.

Waldmann TA, Intestinal lymphangiectasia. In: Birth defects compendium, 3rd Ed, in press.

Waldmann TA, Goldman C, Tsudo M. Aberrant expression of the multisubunit interleukin=2 receptor in HTLV-I-induced adult T cell leukemia. In: Nakamura JM, Diwan A, eds. Proc Asia-Pacific Conference on Human Retroviral Infections. Honolulu: Univ Hawaii, in press.

Waldmann TA, Goldman C, Top L, Grant A, Burton J, Bamford R, Roessler E, Horak I, Zaknoen S, Kasten-Sportes C, White J, Nelson D. The interleukin-2 receptor: a target for immunotherapy. Gupta S, Waldmann TA, eds. In: Mechanisms of lymphocyte activation and immune regulation IV. Proc of 4th Int'l Conf on Lymphocyte Activation and Immune Regulation. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04015-3-MET

PERIOD COVERED: October 1, 1991 through September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Development and Function of Humoral and Cellular Immune Mechanisms

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

PI:	R. Michael Blaese	Senior Investigator	MET, NCI
	Kenneth Culver, M.D.	Medical Staff Fellow	MET, NCI
	Kimberly Leichtling, Ph.D.	Special Volunteer	MET, NCI
	Craig Mullen, M.D., Ph.D.	Medical Staff Fellow	POB, NCI
	Edward Oldfield, M.D.	Chief	SNB, NINDS

COOPERATING UNITS *(if any)*

W. French Anderson, M.D.	Chief	MHB, NHLBI
Steven A. Rosenberg, M.D., Ph.D.	Chief	SB, NCI
Gene Shearer, Ph.D.	Section Chief	EIB, NCI

LAB/BRANCH: Metabolism Branch

SECTION: Cellular Immunology

INSTITUTE AND LOCATION: DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8	4	4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither 100%

☒ (a1) Minors

☐ (a2) Interviews

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

Michael Blaese's laboratory continues to focus on the development of gene therapy. He led the group which performed the first authorized use of gene transfer to treat human disease when they infused 10^9 autologous ADA gene-corrected T cells into a 4-yr-old girl with ADA deficiency SCID. Retroviral vectors were used to insert a normal human ADA gene into this girl's 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 expanded 100-1000 fold and then returned intravenously within 2 weeks to maintain a polyclonal repertoire. This girl and a second ADA deficient patient have been treated 10-12 times over the past two 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. 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. In addition to our ongoing clinical and laboratory evaluation of other immunodeficiency disorders such as the Wiskott-Aldrich syndrome, work has also continued on the development of succinylacetone (SA) as a clinically useful immunosuppressive compound. We have shown that SA has profound dual system immunosuppressive effects in rats, mice, dogs, miniature swine and non-human primates. It is effective in preventing allograft rejection, graft vs host disease (GVHD), antibody production in these experimental animal models and has shown utility in treating several different autoimmune disorders including experimental autoimmune uveitis and adjuvant arthritis.

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

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. In addition, immune T cells can be very long lived as evidenced by the observation that adults maintain DTH and antibody to antigens such as tetanus toxoid for decades after their initial immunization. We first demonstrated that the hADA gene could be introduced into antigen-specific murine CD4 T cells in vitro and that these gene-modified cells would persist in recipient mice for several months and continue to express the introduced hADA gene. We then showed that T cells cultured from monkey blood or lymph node could be successfully transduced with a foreign gene and that these gene-modified T cells would persist for up to 2 years when reintroduced into the autologous monkeys.

As an initial application of gene transfer in a clinical situation based on these findings, we established a collaboration with Steven Rosenberg of the NCI Surgery Branch. This gave us the opportunity to evaluate the consequences of retrovirus mediated gene transfer in patients with terminal cancer and a limited life expectancy who were already being treated with lymphocyte infusions. Our study used the NeoR gene to label tumor infiltrating lymphocytes (TIL) so that their survival and distribution in the body could be determined to see if this might correlate with the anticancer effect. We were able to show that TIL remain in the peripheral blood for about 3 weeks after a single iv infusion and that they localize to the sites of tumor metastases within 2-3 days in patients who experience subsequent remission. Importantly, these studies also demonstrated that retroviral-mediated gene transfer into lymphocytes could be successfully employed in patients and that no untoward consequences at all were observed in the recipients of the gene-modified cells.

With the experience of this successful clinical application of gene transfer behind us, we next moved on to the initial use of gene transfer for the treatment of human disease, true gene therapy. In our studies of children with ADA deficiency SCID, it was shown that unexpectedly we grew polyclonal T cells from their peripheral blood if a combination of anti-TCR monoclonal antibody (OKT3) and IL2 was used to stimulate T cell proliferation. We also demonstrated that we could successfully insert the corrective ADA gene into these proliferating non-transformed T cells using retroviral vectors and that the inserted gene was expressed and the transduced cells produced normal quantities of adenosine deaminase enzyme which was functionally active. On September 14, 1990, the first authorized gene therapy experiment began with the treatment of a 4-year-old girl with ADA deficiency. Subsequently a second child has been enrolled in the protocol and both are doing very well. The patient's T cells are collected periodically from their peripheral blood by apheresis, cultured to expand their number by 100-1000 fold while the ADA gene is inserted, and then reinfused intravenously. To date, the first patient has received 10 infusions and the second patient 11 treatments. The peripheral T cell count is now in the normal range for each child. Each is now also producing normal amounts of antibodies to red blood cells (isohemagglutinins), responses which were deficient before treatment began and enhanced T-cell function and DTH. We will continue to extensively evaluate the immune function in these patients over the next several years as well as enroll additional patients into the study in the coming months. A similar strategy of cellular immunotherapy will be studied in patients with AIDS in the next year. Here, peripheral T cells will be gene-modified to introduce resistance to retroviral replication, etc., culture expanded and then reinfused into the patients. A series of different gene modifications are planned for these studies. 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 for herpes simplex thymidine kinase, he has shown cure of brain tumors in rats following systemic administration of Ganciclovir.

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.

Publications:

Blaese RM. Developments in the application of gene therapy to immunodeficiency and cancer. Proceedings of the 21st Wayne State University Cancer Symposium, in press.

Blaese RM. Gene therapy. The World Book Encyclopedia 1992;8:82.

Blaese RM. Lymphocytes for gene therapy. In: Cohen-Haguenauer O, Boiron M, eds. Human gene transfer. INSERM/John Libbey Eurotext 1991;219:137-45.

Blaese RM. Progress toward gene therapy. J Clin Immunol Immunopath 1991;61:S47-S55.

Blaese RM, Culver KW. Progress toward the application of gene therapy. In: Nance SJ, ed. Clinical and basic science aspects of immunohematology. Arlington, Va: American Assoc Blood Banks 1991;1-11.

Blaese RM, Culver K. Prospects for gene therapy of human disease. Allergol Immunopathol (Madr) 1991;19:25-8.

Cobbs CS, Malech HL, Leto TL, Freeman SM, Blaese RM, Gallin JI, Lomax KJ. Retroviral expression of recombinant p47phox protein by EBV-transformed B lymphocytes from a patient with autosomal chronic granulomatous disease. Blood 1992;79:1829-1835.

Culver KW, Anderson WF, Blaese RM. Gene Therapy. Encyclopaedia Britannica, in press.

Culver KW, Anderson WF, Blaese RM. Lymphocyte gene therapy. Human Gene Therapy 1991;2:107-09.

Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. In vivo gene transfer with retroviral vector producer cells for treatment of experimental brain tumors. Science, in press.

Mullen CA, Kilstrup M, Blaese RM. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a new negative selection system. Proc Natl Acad Sci USA 1992;89:33-7.

Parenteau GL, Hess RA, Mendeloff EN, Clark RE, Blaese RM. Succinylacetone attenuates rejection of heterotopic cardiac allografts in miniature swine. Surg Forum 1991;42:401-03.

Stone CD, Hess RA, Blaese RM, Clark RE. Succinylacetone: a powerful new non-toxic immunosuppressive for organ transplantation. Surg Forum 1990; 40:339-31.

Winkelstein A, Hess RA, Blaese RM. Inhibition of human lymphoproliferative responses and altered lymphocyte membrane phenotype by succinylacetone. Immunopharmacology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04016-19 MET

PERIOD COVERED

October 1, 1991 through September 30, 1992

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

Mechanism of Action of Insulin-like Growth Factors

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

PI:	S. Peter Nissley, M.D.	Senior Investigator	MET, NCI
	Daisy De Leon, M.D.	Senior Staff Fellow	MET, NCI
	Wlodzimierz Lopaczynski, M.D.	Fogarty Visiting Associate	MET, NCI

COOPERATING UNITS *(if any)*

Bone Cell Biology Section, National Institute of Dental Research

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither "B" 100%

☐ (a1) Minors

☐ (a2) Interviews

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

We have developed a rapid, sensitive dot-blot assay to screen transfected cell lines for the production of insulin-like growth factor-II (IGF-II). This chemiluminescence dot-blot method detects picogram quantities of IGF-II which is 100 times more sensitive than conventional IGF-II radioimmunoassays and can be completed in 5-6 hrs.

We have asked whether or not the IGF-I receptor utilizes the phosphatidylinositol pathway in MG63 human osteosarcoma cells by measuring levels of diacyglycerol (DAG) following addition of IGF-I. No increase in DAG over levels in unstimulated cells was seen at 2,5,10, and 30 min following addition of IGF-I. We conclude that the IGF-I receptor does not signal biologic responses to IGF-I by stimulating the hydrolysis of phosphatidylinositol or phosphatidylcholine.

We have examined IGF-I receptor expression and function in fibroblasts from 2 patients with deletion of the distal long arm of chromosome 15. It had been proposed by others that the severe growth impairment seen in patients with 15 q deletion syndrome may be related to the loss of a single copy of the IGF-I receptor gene. Quantitative Southern blot analysis of Hind III digests of fibroblast DNA showed that the IGF-I receptor band was 41.3 $\pm$ 13% and 43.5 $\pm$12% compared to the control fibroblasts, suggesting loss of one copy of the IGF-I receptor gene in the patients. Cell surface expression of the IGF-I receptor was assessed by binding of the IGF-I analog, ^{125}I-long R^{3}IGF-I to fibroblast monolayer cultures. Using the amount of tracer binding that was blocked by the IGF-I receptor monoclonal antibody, αIR-3, as a measure of binding to the IGF-I receptor, the patients fibroblasts showed significantly lower cell surface expression of the IGF-I receptor. To assess receptor function, stimulation of N-methyl-[^{14}C]α-aminoisobutyric acid uptake by a full range of IGF-I concentrations was examined in serum-starved cultures. There were no significant differences between the patient and control fibroblasts. We conclude that although there is evidence for decreased cell surface expression of the IGF-I receptor in fibroblasts that are missing one copy of the IGF-I receptor gene, the stimulation of amino acid uptake by IGF-I is not impaired.

Project Description

Major Findings:

Development of a rapid, sensitive dot-blot assay to screen transfected cell lines for the production of insulin-like growth factor-II (IGF-II)

In order to test our hypothesis that endogenous IGF-II could modulate the cellular trafficking of lysomal enzymes by the mannose 6-phosphate/IGF-II receptor by inhibiting binding to the receptor, we plan to transfect a human breast cancer cell line (MCF-7) with the human IGF-II gene and assess the distribution of newly synthesized lysosomal enzymes between the extracellular and intracellular compartments. The identification of transfected clones that produce large amounts of IGF-II requires a convenient method for the rapid detection of IGF-II in small amounts of conditioned media from multiple samples. Conventional radioimmunoassay methods are insensitive and require the separation of IGF-II from IGF binding proteins prior to assay. We have developed a dot-blot assay based on the Amersham enhanced chemiluminescence (ECL) methodology. Samples of conditioned media or IGF-II standard are blotted onto nitrocellulose filter paper. The blots are incubated sequentially in IGF-II monoclonal antibody, biotinylated secondary antibody, and streptavidin-biotinylated-horseradish peroxidase complex. Added luminol is oxidized by a horseradish peroxidase catalyzed reaction, emitting light which is enhanced and detected by very brief exposure to a film. This chemiluminescence dot-blot method detects picogram quantities of IGF-II which is 100 times more sensitive than conventional IGF-II radioimmunoassays and can be completed in 5-6 hrs.

The role of hydrolysis of phoshatidylinositol in signaling by the IGF-I receptor in MG63 human osteosarcoma cells

A number of growth factors have been shown to stimulate the rapid hydrolysis of plasma membrane phosphatidylinositol leading to the rapid increase in intracellular inositol phosphates (IP) and diacylglycerol (DAG). IP3 mobilizes intracellular Ca^{++} and DAG activates protein kinase C. The human osteosarcoma cell line, MG63, has characteristics which make it an attractive model system to study the growth promoting action of IGF-I. MG63 cells multiply in serum-free medium with IGF-I as the only growth factor, and growth stimulation is mediated by the IGF-I receptor. We asked whether the signaling of the IGF-I-stimulated growth response in MG63 cells utilizes the phosphatidylinositol pathway. We added IGF-I to growth-arrested cells and measured DAG levels. Lipid was extracted from the cell monolayers and DAG was measured with an enzymatic assay which utilizes DAG kinase and [γ-^{32}P]ATP to convert DAG to [^{32}P]phosphatidic acid. The reaction product was analysed by thin-layer chromatography and the [^{32}P]phosphatidic acid spot was scrapped from the plate and radioactivity measured in a scintillation counter. The plates were also scanned with a PhosphorImager as an alternative method to measure relative radioactivity in the [^{32}P]phosphatidic acid spot. Results were expressed as mass of DAG by including a DAG standard in the assay. No increases of DAG over levels in unstimulated MG63 cells were seen at 2, 5, 10, and 30 min after addition of IGF-I. As a positive control we confirmed that α-thrombin stimulated a several fold increase in DAG in a Chinese hamster fibroblast cell line (IIC9). We conclude that unlike other growth factor receptors (EGF, PDGF, bombesin) the IGF-I receptor does not utilize the phosphatidylinositol pathway. Since DAG can also arise from hydrolysis of phosphatidylcholine, the IGF-I receptor also does not signal the hydrolysis of phosphatidylcholine by phospholipase C.

Insulin-like growth factor-I receptor expression and function in fibroblasts from patients with deletion of the distal long arm of chromosome 15

Most of the patients with deletions of distal 15q have intrauterine growth retardation (IUGR) and postnatal growth deficiency in addition to other developmental abnormalities. Recently, Roback and colleagues have reported the loss of one IGF-I receptor gene copy in an infant with a deletion of

chromosome 15 (15q 26.1→qter). We have examined IGF-I receptor expression and function in fibroblasts with deletion of the distal long arm of chromosome 15, provided by Dr. Richard Kelley, Johns Hopkins Hospital. Both patients have features of the 15q deletion syndrome including severe IUGR in a full-term female and a height of less than 6 SD below the mean in an 11-year-old female. For all studies, the patients' fibroblasts were compared with skin fibroblasts from 2 age-matched controls. Quantitative Southern blot analysis of the IGF-I receptor gene was performed on Hind III digests of fibroblast DNA using as a probe a 0.7 kb Eco RI fragment of the cDNA for the human IGF-I receptor (IGF-I-R.8, ATCC, A. Ullrich). A genomic probe for a more proximal position on chromesome 15q was used as a control. The radioactivity of the IGF-I receptor band (1.7 kb) and the control band (1.2 kb) was measured with a PhosphorImager. The IGF-I receptor band in the patients was 41.3 +/- 13% and 43.5 +/- 12% of the control fibroblasts and the control band in the patients was 81.1 +/- 22% and 87.5 +/- 16% of the control fibroblasts ($P = 0.003$), suggesting loss of one copy of the IGF-I receptor gene in the patients. Affinity crosslinking experiments with ^{125}I-IGF-I showed that the IGF-I receptor was present in the patients' fibroblasts and was normal size.. Cell surface expression of the IGF-I receptor was assessed by binding of the IGF-I analog, ^{125}I-long R^3IGF-I to serum-starved monolayer cultures at 4 C. (Long R3IGF-I was chosen because of greatly decreased binding to IGF binding proteins.) Using the amount of tracer binding that was blocked by the IGF-I receptor antibody αIR-3 as a measure of binding to the IGF-I receptor, the patients fibroblasts showed lower cell surface receptor expression (180 +/- 106 cpm versus 669 +/- 370 cpm, $P < 0.01$). IGF-I stimulation of the transport of the nonmetabolizable amino acid, N-methyl-α-aminoisobutyric acid through the A transport system is signaled by the IGF-I receptor. To assess receptor function, stimulation of N-methyl-[^{14}C]α-aminoisobutyric acid uptake by a full range of IGF-I concentrations was examined in confluent serum-starved monolayer cultures. There were no differences between the patient and control fibroblasts (ED50:2.2 +/- 1.5 ng/ml versus 2.2 +/- 0.7 ng/ml; fold stimulation over basal:2.6 +/- 1.0 versus 2.8 +/- 1.7: maximal uptake/20 min: 9938 +/- 2996 cpm versus 7392 +/- 1982 cpm). We conclude that although there is evidence for decreased cell surface expression of the IGF-I receptor in fibroblasts that are missing one copy of the IGF-I receptor gene, the stimulation of amino acid uptake by IGF-I is not impaired.

Publications:

Nissley P. Insulin-like growth factor receptors, pp856-57. In: LeRoith D, moderator. Insulin-like growth factors in health and disease. Ann Intern Med 1992;116:854-62.

Nissley P, Kiess W. Reciprocal modulation of binding of lysosomal enzymes and insulin-like growth factor II (IGF-II) to the mannose 6-phosphate/IGF-II receptor. In: Raizada MK, LeRoith D, eds. Molecular biology and physiology of insulin and insulin-like growth factors. Advances in Experimental Medicine and Biology Vol 293. New York: Plenum Press 1991;311-24.

Nissley SP, Kiess W. Binding of insulin-like growth factor-II (IGF-II) and lysosomal enzymes to the IGF-II/mannose-6-phosphate receptor. In: Spencer EM, ed. Modern Concepts of Insulin-like Growth Factors. New York: Elsevier 1991;419-30.

Nissley SP, Lopaczynski W. Insulin-like growth factor receptors. Growth Factors 1991;5:29-43.

Sklar MM, Thomas CL, Municchi G, Roberts CT, LeRoith D, Kiess W, Nissley SP. Developmental expression of rat insulin-like growth factor-II/mannose 6-phosphate receptor messenger ribonucleic acid. Endocrinology 1992; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04017-14 MET

PERIOD COVERED: October 1, 1991 through September 30, 1992

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)
Biology of the Immune Response

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

PI:	David L. Nelson, M.D.	Head	MET, NCI
	Jeffrey D. White, M.D.	Medical Staff Fellow	MET, NCI
	Glen Bock, M.D.	IPA	MET, NCI
	Hariclia Litou, M.D.	Visiting Fellow	MET, NCI
	S. Bhagovati, M.D.	Expert	MET, NCI

COOPERATING UNITS (if any)

LAB/BRANCH: Metabolism Branch

SECTION: Immunophysiology

INSTITUTE AND LOCATION: DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.8	5	1.8

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☒ (a2) Interviews

"B" 100%

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

Studies were performed to examine the maturation and regulation of the human immune response in normal individuals and in patients with congenital and acquired immune deficiency states associated with a high frequency of cancer. The interaction of the T-cell derived lymphokine interleukin-2 with its cell membrane receptor (IL-2R) plays a pivotal role in the generation of immune responses. We have identified a soluble form of the IL-2R (sIL-2R) in the serum of normal individuals and found elevated levels of this receptor in a variety of malignancies of the lymphoreticular system. In patients with the Adult T-cell Leukemia (ATL), reductions in serum levels of sIL-2R correlated with responses to chemotherapy. In collaboration with Dr. Thomas Waldmann, this has also been shown in ATL patients receiving IL-2R directed therapies. Elevated serum levels of sIL-2R were also observed in patients with the acquired immune deficiency syndrome (AIDS) and carriers of the Human Immunodeficiency Virus type 1. Thus the measurement of sIL-2R is useful in the management of patients with immunologic activation in vivo. Another T-cell derived lymphokine, interleukin-6 (IL-6) plays a pivotal role in B-cell maturation. We have recently established an IL-6 responsive human tumor cell line which shares many features with the lymphoreticular malignancies occurring in AIDS patients. Approaches to the diagnosis and treatment of AIDS lymphomas using this cell line are currently underway.

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.

Publications:

Bhoopat L, Scollard DM, Theetranont C, Chiewchanvit S, Nelson DL, Utaipat U. Studies of human leprosy lesions in situ using scution-induced blisters: Cell changes with IgM antibody to PGL-1 and interleukin-2 receptor in clinical subgroups of erythema nodusum leprosum. Asian Pacific J of Allergy and Immunology 1991;9:107-119.

Boumpas DT, Anastassiou ED, Older SA, Tsokos GC, Nelson DL, Balow JE. Dexamethasone inhibits human interleukin-2 receptor gene expression in vitro at the level of nuclear transcription. J Clin Invest 1991;87:1739-47.

Engler RJM, Kurman CC, Nelson DL. Induction and measurement of in vivo antibody responses. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach E M, Strober W, eds. Current Protocols in Immunology. New York: John Wiley & Sons 1991;7.16.1-7.16.7.

Kurman CC, Rubin LA, Nelson DL. Soluble products of immune activation: soluble interleukin-2 receptor (sIL-2R, Tac protein). In: Rose NR, deMacario E C, Jahey JL, Friedman H, Penn GM, eds. Manual of Clinical Laboratory Immunology. Washington DC: American Society for Microbiology 1992;256-260.

Kurman CC, Rubin LA, Nelson DL. Soluble interleukin-2 receptor (sIL-2R, Tac protein). In: Rose NR, ed. Manual of Clinical Laboratory Immunology, in press.

Nelson, D. L., Kurman, C. C., Notarangelo, L. D. The Cells and Molecules of the Immune System. Prospective in Pediatrica. 85:5-11, 1992.

Nelson DL, Kurman CC. The human immune response: molecular recognition and immunoregulation. In: Sacher RA, Brubaker DB, Kasprisin D, McCarthy LJ, eds. Apheresis in Cellular and humoral immunotherapy and apheresis. Arlington, VA: American Assoc of Blood Banks 1991;61-72.

Plebani A, Notarangelo LD, Parolini O, Duse M, Nelson D, Ugazio AG. Demonstration of abnormalities of major histocompatibility complex Class III genes in a family with IgA deficiency. In: Chapel HM, Levinsky RJ, Webster A DB, eds. Progress in Immune Deficiency III. London: Royal Society of Medicine Services Limited 1991;41-42.

Rapaport MH, Doran AR, Nelson DL, McAllister C, Magliozzi JR, Paul SM. Haloperidol and soluble interleukin-2 receptors. [letter] Biol Psychiatry 1991;30:1063-4.

Silverman ED, Laxer RM, Nelson DL, Rubin LA. Soluble interleukin-2 receptor in juvenile rheumatoid arthritis. J Rheumatol 1991;18:1398-402.

Young JB, Lloyd KS, Windsor NT, Cocanouger B, Weilbaecher DG, Kleiman NS, Smart FW, Nelson DL, Lawrence EC. Relationship of elevated soluble interleukin-2 receptor levels measured early after heart transplant to long-term survival and development of coronary arteriopathy. J Heart and Lung Transpl 1991;10:243-50.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04018-16 MET

PERIOD COVERED: October 1, 1991 through September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Immunoregulatory Glycoproteins: Purification and Characterization

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

PI:	Andrew V. Muchmore, M.D.	Senior Investigator	MET, NCI
	Bibhuti Mishra, M.D.	Medical Staff Fellow	MET, NCI
	Jean Decker, B.S.	Chemist	MET, NCI
	Michael Dipre, M.D.	Medical Staff Fellow	MET, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Metabolism Branch

SECTION: Cellular Immunology

INSTITUTE AND LOCATION: DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4½	4	½

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither "B" 100%

☐ (a1) Minors

☐ (a2) Interviews

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

Dr. Muchmore's interest in the functional role of defined glycopeptides and oligosaccharides as regulatory signals in their own right has led to a clearer appreciation of the physiologic role of these structures in regulating the cell cycle and state of cell activation as well as the view that these compounds represent a new class of agents with broad pharmaceutical activities. Dr. Mishra, working with Dr. Muchmore extended previous observations to implicate a common mechanism for the activity of these pluripotent glycopeptides. Their data demonstrate that high mannose glycopeptides in structurally specific fashion act directly in the nucleus to regulate gene transcription. Using the AP-1 model first described in HeLa cells, Dr. Muchmore showed that Man-5 phosphate blocks the ability of the heterodimeric transcription factors fos-jun to bind to their consensus DNA binding region. These observations have been expanded to show a functional effect not only on in vitro transcription but also on intact cellular proliferation. Specific mannose structures block the unbridled proliferation of HeLa cells and at least partially induce contact growth inhibition. This ability to apparently reverse the malignant phenotype of HeLa cells suggests that these studies offer an entirely new avenue of research in efforts to understand and control malignant cell growth. In addition studies with transfected fibroblast lines from patients show that disorder of CNS development in mannosidosis is probably caused by inhibition of AP-1-mediated gene transcription.

In related studies Dr. Muchmore has defined a high affinity receptor specific for uromodulin, an inhibitor of lymphocyte proliferation. Furthermore, he has isolated and partially sequenced a cDNA clone from a human testis library which represents the human homologue of a sea urchin sperm protein known to have homology to uromodulin and to be functionally important in acrosomal body formation.

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

Project Description

Our laboratory's interest in the functional role of defined glycopeptides and oligosaccharides as regulatory signals in their own right, has led us to describe their effect on growth and proliferation of malignant cells. These studies have led not only to a much clearer fundamental appreciation of the physiologic role of these structures in regulating the cell cycle and state of cell activation, but also led us to the exciting conclusion that these compounds represent a totally new class of agents with broad pharmaceutical activities. Earlier work from our laboratory has demonstrated that defined glycoproteins may play an important role during human pregnancy (Science 1985, 1987) as well as regulating a host of cellular activities including cytotoxicity (Cancer Research 1990) PGE2 synthesis (Mol and Cell Biochem 1991) and the activity of human cytokines (J Biol Chem 1988, J Immunol 1989). Dr. Mishra in our laboratory has extended these observations to implicate a common mechanism for the activity of these pluripotent glycopeptides. Our data demonstrate that high mannose glycopeptides in an absolutely structurally specific fashion act directly in the nucleus to regulate gene transcription. Using the AP-1 model first described in HeLa cells, he has shown that Man-5 blocks the ability of the heterodimeric transcription factors fos-jun to bind to their consensus DNA binding region. These observations have been expanded to show a functional effect not only on in vitro transcription, but much more importantly on intact cellular proliferation. Specific mannose structures block the unbridled proliferation of HeLa cells and at least partially induce contact growth inhibition. This ability to apparently reverse the malignant phenotype of HeLa cells strongly suggests that these studies offer an entirely new avenue of research in our efforts to understand and control malignant cell growth. In addition, studies with transfected fibroblast lines from patients show that disorder of CNS development in mannosidosis is probably caused by inhibition of AP-1-mediated gene transcription.

Our laboratory has also pursued two other projects. The first concerns the mechanism of inhibition of lymphocyte proliferation by uromodulin. We have defined a high affinity receptor specific for uromodulin (approx. 2×10^5 receptors/cell with a kD of approximately 10^{11}M. Expression of a T-cell CDNA library in the expression system lambda gt-11 has yielded several promising clones which have been purified following a secondary screening procedure. Initial sequencing of these clones interestingly shows a strong homology to uromodulin itself but does not share identity with any previously sequenced genes. The second project deals with the function of an emerging uromodulin family. We have isolated and partially sequenced a cDNA clone from a human testis library which represents the human homolog of a sea urchin sperm protein know to have homology to uromodulin and to be functionally important in acrosomal body formation.

Major Findings:

1. Mannose-5, purified from ovalbumin inhibits specific DNA binding by AP-1 and jun. This inhibition is structurally specific. It is not seen when using other high mannose glycopeptides or Man-5 with other transcription factors.

2. Inhibition of DNA binding leads to inhibition of transcription. Using CAT and gel shift assays with mannosidase-deficient cell lines, we have evidence that this inhibition may be responsible for disordered development in mannosidosis.

3. We have found a mannose-binding protein which shows a 38% identity with the DNA binding region of C-jun.

4. Man-5 directly inhibits the growth characteristics of the malignant cell line HeLa. We believe this effect is modulated by its effect on AP-1.

5. Using a cDNA library from human sperm we have cloned and perhaps characterized another member of the uromodulin family of proteins. This member was originally discovered in sea urchin sperm.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04020-15 MET

PERIOD COVERED
October 1, 1991 through September 30, 1992

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)

PI: Jay A. Berzofsky, M.D., Ph. D.	Section Chief	MET, NCI
Toshiyuki Takeshita, M.D., Ph.D.	Visiting Fellow	MET, NCI
Richard England, M.D., Ph.D.	Medical Staff Fellow	MET, NCI
Mutsunori Shirai, M.D., Ph.D.	Visiting Associate	MET, NCI
Marika Kullberg	Special Volunteer	MET, NCI
Michael Yanuck	HHMI Scholar	MET, NCI

COOPERATING UNITS (if any)

Gene M. Shearer, Ph.D.	Section Chief	EIB, NCI
Richard Hodes, M.D.	Section Chief	EIB, NCI
Melanie Vacchio, Ph.D.	Postdoctoral Fellow	EIB, NCI

LAB/BRANCH
Metabolism Branch (More professional personnel listed on next page)

SECTION
Molecular Immunogenetics and Vaccine Research Section

INSTITUTE AND LOCATION
DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10.5	8.5	2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors "B" 100

☐ (a2) Interviews

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

We studied mechanisms by which T cells recognize antigens with major histocompatibility complex (MHC)-encoded molecules, and applications to the design of synthetic vaccines for AIDS and cancer. We characterized a peptide fragment of the HIV-1 envelope recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) using a new system we developed in which CTL are stimulated by peptide bound to purified Class I MHC molecules on plastic. A 10-residue HIV peptide is a million times more active than the original 15-residue peptide in the absence of serum, and the longer peptide requires processing by angiotensin converting enzyme (ACE) in serum. We also showed that the Class I molecule plays two roles in CTL activation, one to present the peptide and the other probably to interact with CD8, as it requires only a conserved alpha 3 domain. The MHC molecule binding CD8 does not have to be the same one presenting peptide. We have shown that non-crossreactive CTL distinguish aliphatic from aromatic residues at a single position in this peptide. This finding led us to discover a way to induce broadly crossreactive CTL against multiple variants of HIV by stimulation with a chimeric peptide. CTL fine specificity correlated with specific receptor variable regions used. This CTL site, which also is a target of neutralizing antibodies, has been coupled to sites we identified that stimulate T helper cells in mice and humans of multiple MHC types, and the resulting candidate synthetic vaccine has been found to induce extremely high titers of neutralizing antibodies in mice, as well as specific CTL killing of HIV-1 envelope expressing cells. Also, an early diagnostic test is being developed. We also showed that schistosomiasis resulted in reduced clearance of concurrent vaccinia virus infection and decreased IL-2, interferon and CTL responses. This may account in part for the rapid spread of AIDS in Africa. We are also attempting cancer vaccines to induce CTL to mutant peptides corresponding to oncogene mutations that could kill tumor cells. We succeeded in inducing peptide-specific CTL that will kill tumor targets expressing a mutant p53 gene. Thus such mutant oncogene products, although not expressed on the cell surface, can serve as targets of specific cancer immunotherapy. Also, a CTL determinant of the hepatitis C virus has been found.

Cooperating Units:

Peter Nara, DVM	Section Chief	LTCB, NCI
Louis H. Miller, M.D.	Lab Chief	LMR, NIAID
Ronald N. Germain, M.D., Ph.D.	Section Chief	LI, NIAID
David Margulies, M.D., Ph.D.	Senior Investigator	LI, NIAID
Steve Kozlowski, M.D.	Fellow	LI, NIAID
Alan Sher, M.D.	Section Chief	LPD, NIAID
Jeffrey Actor, Ph.D.	Postdoctoral Fellow	LPD, NIAID
Mark Buller, M.D.	Senior Investigator	LVD, NIAID
Bernard Moss, M.D., Ph.D.	Lab Chief	LVD, NIAID
Mario Clerici, M.D.	Visiting Fellow	EIB, NCI
Sanjai Kumar, Ph.D.	Staff Fellow	LMR, NIAID
Stephen Feinstone, M.D.	Senior Investigator	CBER, FDA
Peter Howley, M.D.	Lab Chief	LTVB, NCI
Stephen Hoffman, M.D.	Cdr.	ID, NMRI
Walter Weiss, M.D.	Lt. Cdr.	ID, NMRI

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.

We have characterized a peptide fragment of the HIV-1 envelope protein gp160 recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) using a new system we developed in collaboration with David Margulies and Steve Kozlowski in which CTL are stimulated by peptide bound to purified soluble recombinant Class I MHC molecules attached to plastic microtiter wells, and responses measured as interferon-gamma production. We originally identified a 15-residue peptide of HIV-1 gp160 that was immunodominant for recognition by CTL from $H\text{-}2^d$ mice, and that was presented by the Class I molecule D^d. In mapping the minimal determinant, we found that a 10-residue HIV peptide contained within the 15-mer was a million times more active than the original 15-residue peptide in the absence of serum, and the longer peptide required proteolytic processing by angiotensin converting enzyme (ACE) in serum. Presentation of the 15-mer in serum was inhibited by the ACE-inhibitor captopril, but 15-mer pretreated with purified ACE was presented without serum, as was the untreated 10-mer.

We also showed that the Class I molecule plays two roles in CTL activation, one to present the peptide and the other probably to interact with CD8, as it requires only a conserved alpha 3 domain. Simultaneous titration of MHC molecules on the plastic and peptide concentration showed a higher order dependence of the response on the MHC concentration than on the peptide concentration. This order of dependence was reduced in half if the purified D^d was titrated in the presence of a compensating amount of recombinant K^b Class I molecule that cannot present the peptide but has a shared alpha 3 domain that can interact with CD8 on the T cell, so that the total Class I molecule concentration was held constant. Thus, the MHC molecule binding CD8 does not

have to be the same one presenting peptide. We are also using this system to quantitate requirements for CTL activation, and to determine kinetics of functional stability of the peptide-MHC molecule complex.

We have characterized the fine specificity of CTL for this HIV-1 gp160 peptide in collaboration with Hidemi Takahashi, a lab alumnus, and shown that non-crossreactive CTL specific for different isolates of HIV such as IIIB and MN distinguish aliphatic from aromatic residues at a single position (residue 325). IIIB-specific CTL recognize variants with any aliphatic residue at this position, e.g., Leu, Val, or Ile, whereas MN-specific CTL recognize variants with any aromatic residue at this site, e.g., Tyr, Phe, Trp, His, as well as the cyclic non-aromatic residue Pro. Thus the T-cell receptor makes a broad distinction in the chemical nature of the side chain. This finding led us to discover a way to induce broadly crossreactive CTL against multiple variants of HIV by stimulation with a chimeric peptide, resembling the MN sequence except for an aliphatic residue at position 325. CTL induced in this way recognized not only sequences with aliphatic and aromatic residues at 325, but also recognized ones with charged residues such as Lys and Arg, and uncharged polar residues such as Gln, that are not recognized by either IIIB- or MN-specific CTL. These crossreactive CTL also recognize a broader range of natural isolates of HIV, and so a similar approach in humans may allow induction of immunity to multiple isolates and help prevent escape mutation.

This same HIV peptide was also found to crossreact with a superficially unrelated peptide from the gp41 region of the HIV envelope, even when presented by 4 different Class I MHC molecules that can present both peptides. The crossreaction was not due to a mixture of peptides, as shown by use of multiple preparations, and by studying other functional responses in which the peptides do not crossreact. It was also not due to a mixture of CTL, because cold-target blocking showed that targets expressing one peptide could inhibit recognition of targets expressing the other peptide, and vice versa, and because the crossreactivity held when monoclonal CTL were prepared. The crossreactive region was mapped to a single 10-residue core peptide presented by all 4 MHC molecules. The crossreactive CTL of 3 distinct MHC types use a limited repertoire of receptor variable regions, Vβ8 and Vβ14, whereas the non-crossreactive CTL do not use these receptors ($p < 0.005$).

To develop a candidate synthetic vaccine for HIV, we have coupled this CTL target peptide, which also is a target of neutralizing antibodies, to multideterminant peptides we identified that stimulate T helper cells in mice and humans of multiple MHC types. Three resulting candidate synthetic vaccines have been found to induce extremely high titers of neutralizing antibodies in mice of multiple MHC types, as well as specific CTL killing of HIV-1 envelope expressing cells. We are currently characterizing the neutralizing antibodies and comparing these with non-neutralizing antibodies to the same peptide to determine whether the difference in function is due to a difference in affinity, fine specificity, or isotype. We are also comparing the candidate constructs to find the optimal one for an immunotherapeutic trial, and are comparing different adjuvants for antibody and CTL induction. A Phase I human immunotherapy trial with one of these constructs is being planned.

In collaboration with Gene Shearer and Mario Clerici, we have studied the responses of peripheral blood T cells, measured as IL-2 production, to some of our helper T-epitope peptides from HIV-1 gp160 in individuals who had known exposure to HIV-1 but who have remained seronegative. Five of five exposed individuals responded to at least four of five peptides tested, whereas only seven of 136 low-risk seronegative controls responded to any HIV peptides, and only two of these responded to more than one peptide. One of the five exposed individuals later seroconverted, but the others have remained seronegative and PCR-negative. Although we cannot document that these others are infected, the T-cell response seems to be an earlier diagnostic measure of exposure, if not infection, than the antibody response, and may have clinical application.

In another aspect of AIDS research, we have collaborated with Alan Sher to examine the effect of parasitic infection, as is rampant in Africa, on other immune responses. We initially observed that

mice infected with *Schistosoma mansoni* altered their responses to unrelated antigens to which they were exposed during the parasite egg-laying stage. The T-cell response to myoglobin was shifted from Th1 to Th2 phenotype; that is, IL-2 and interferon-gamma were suppressed and IL-4 enhanced. To see if this effect applied to viral infections acquired during schistosomiasis, we infected mice with schistosomiasis and then with a vaccinia virus recombinant expressing HIV envelope and found that they made reduced IL-2, interferon, and CTL responses specific for HIV envelope and vaccinia, and had delayed elimination of the virus from tissues. This may account in part for the severity of AIDS in Africa, where such parasites are endemic.

The approach used in our AIDS vaccine research has also been applied to cancer vaccines of two types: 1) vaccines aimed at inducing CTL to mutant peptides corresponding to oncogene mutations (especially in p53 and ras, that are common in many human cancers), that could kill tumor cells; and 2) vaccines aimed at oncogenic viruses, such as human papillomavirus (HPV) and hepatitis C virus (HCV). The former project is in collaboration with John Minna and David Carbone at University of Texas Southwestern Medical Center, and the latter project also includes collaborations with Peter Howley and Bernard Moss at NIH and Steve Feinstone at the FDA. We succeeded in inducing murine CTL to a peptide corresponding to a human p53 tumor suppressor gene mutation, that will kill syngeneic transfected tumor targets expressing the mutant p53 gene. This demonstrates that such mutant oncogene products, although not expressed on the cell surface, can serve as potential targets of specific cancer immunotherapy. Also, a CTL determinant of the cancer-causing hepatitis C virus has been found, and work is underway to study CTL responses to the E6 and E7 oncogene proteins of HPV 16, that causes human cervical carcinoma. Because these viral genes are expressed in the cancer cells, they should make good foreign antigen targets for immunotherapy or prophylaxis of cervical cancer.

Honors and Awards:

31st Michael Heidelberger Award and Lecture, Columbia University, 1992
President-elect, American Society for Clinical Investigation, 1992

Publications:

Berzofsky, JA. Antigenic peptide interaction with MHC molecules: implications for the design of artificial vaccines. Seminars in Immunology 1991;3:203-16.

Berzofsky JA. Approaches and issues in the development of vaccines against HIV. J Acq Immune Defic Syndromes 1991;4:451-59.

Berzofsky, JA. Development of artificial vaccines against HIV using defined epitopes. FASEB J 1991;5:2412-18.

Berzofsky, JA. Human immunodeficiency virus: structural features of T-cell epitopes and their use in vaccine development. In: Thomas DB, ed. Viruses and the cellular immune response. New York: Marcel Dekker 1992; in press.

Berzofsky, J.A. Mechanisms of T cell recognition with application to vaccine design. Molec Immunol 1991;28:217-223.

Berzofsky JA. Progress towards an artificial vaccine for HIV: identification of helper and cytotoxic T-cell epitopes and methods of immunization. Biotech Therapeutics 1991;2:123-35.

Berzofsky JA. Progress towards artificial vaccines for HIV. In: Brown F, Chanock R, Ginsberg H, Lerner R, eds. Vaccines 92. Cole Spring Harbor Laboratory Press, Cold Spring Harbor 1992;41-50.

Berzofsky JA, Pendleton CD, Clerici M, Ahlers J, Lucey DR, Putney SD, Shearer GM. Construction of peptides encompassing multideterminal clusters of human immunodeficiency virus envelope to induce in vitro T-cell responses in mice and humans of multiple MHC types. J Clin Invest 1991;88:876-84.

Berzofsky JA, Pendleton CD, Clerici M, Ahlers J, Lucey DR, Putney SD, Shearer GM. Peptides containing multideterminant clusters of human immunodeficiency virus envelope induce murine and human T-cell responses in diverse histocompatibility types. Transac Assoc Amer Phys 1991;104:69-77.

Clerici M, Berzofsky JA, Shearer GM, Giorgi JV, Tacket C. On HIV-serologic testing of blood and tissue donors. New Engl J Med 1992; in press (letter).

Clerici M, Berzofsky JA, Shearer GM, Tacket CO. Exposure to HIV-1 indicated by HIV-specific T helper cell responses before detection of infection by polymerase chain reaction and serum antibodies. J Infect Dis 1991;164:178-82.

Clerici M, Giorgi JV, Chou C-C, Gudeman VK, Zack JA, Gupta P, Ho H-N, Nishanian PG, Berzofsky JA, Shearer GM. Cell mediated immune response to human immunodeficiency virus type I (HIV-1) in seronegative homosexual men with recent sexual exposure to HIV-1. J Infect Dis 1992;165:1012-19.

Clerici M, Tacket CO, Via CS, Lucey DR, Muluk SC, Zajac RA, Boswell RN, Berzofsky JA, Shearer GM. Immunization with subunit human immunodeficiency virus vaccine generates stronger T helper cell immunity than natural infection. Eur J Immunol 1991;21:1345-49.

Cornette JL, Margalit H, DeLisi C, Berzofsky JA. The amphipathic helix as a structural feature involved in T-cell recognition. In: Epand RM, ed. The amphipathic helix. Boca Raton: CRC Press 1992; in press.

DeGroot AS, Clerici M, Hosmalin A, Hughes SH, Barnd D, Hendrix CW, Houghten RA, Shearer GM, Berzofsky JA. Human immunodeficiency virus (HIV-1) reverse transcriptase T helper epitopes identified in mice and humans: correlation with a cytotoxic T cell (CTL) epitope. J Infect Dis 1991;164:1058-65.

Goodman-Snitkoff G, Good MF, Berzofsky JA, Mannino RJ. Role of intrastructural/intermolecular help in immunization with peptide-phospholipid complexes. J Immunol 1991;147:410-15.

Hosmalin AS, Kumar S, Barnd D, Houghten R, Smith GE, Hughes SH, Berzofsky JA. Immunization with soluble protein-pulsed spleen cells induces class I-restricted CTL that recognize immunodominant epitopic peptides from P. falciparum and HIV-1 J Immunol 1992;149:in press.

Ida H, Kurata A, Eguchi K, Kawakami A, Migita K, Fukuda T, Nakamura T, Kusumoto Y, Berzofsky JA, Nagataki. Different B-cell responses to human T-cell lymphotrophic virus type I (HTLV-I) envelope synthetic peptides in HTLV-I-infected individuals. J Clin Immunol 1991;11:143-51.

Kozlowski S, Corr M, Takeshita T, Boyd LF, Pendleton CD, Germain RN, Berzofsky JA, Margulies DH. Serum angiotensin-1 converting enzyme activity processes an HIV 1 gp160 peptide for presentation by MHC class I molecules. J Exp Med 1992;175:1417-22.

Kullberg MC, Pearce EJ, Hieny SE, Sher A, Berzofsky JA. Infection with Schistosoma mansoni alters Th1/Th2 cytokine responses to a non-parasite antigen. J Immunol 1992;148:3264-70.

Lipham WJ, Redmond TM, Takahashi H, Berzofsky JA, Wiggert B, Chader GJ, Gery I. Recognition of peptides that are immunopathogenic but cryptic: mechanisms that allow lymphocytes sensitized against cryptic peptides to initiate pathogenic autoimmune processes. J Immunol 1991;146;3757-62.

Sher A, Gazzinelli RT, Oswald IP, Clerici M, Kullberg M, Pearce EJ, Berzofsky JA, Mosmann TR, James SL, Morse HC, Shearer GM. Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. Immunological Reviews 1992;127: in press.

Shirai M, Akatsuka T, Pendleton CD, Houghten R, Wychowski C, Mihalik K, Feinstone S, Berzofsky JA. Induction of cytotoxic T cells to a crossreactive epitope in the hepatitis C viral nonstructural RNA polymerase-like protein. J Virol 1992;66:4098-4106.

Shirai M, Berzofsky JA. Promiscuity of cytotoxic T-cell epitopes from the HIV-1 envelope. In: Chanock RM, Ginsberg HS, Brown F, Lerner RA, eds. Vaccines 92. Cold Spring Harbor:Cold Spring Harbor Lab Press 1992;243-46.

Shirai M, Pendleton CD, Berzofsky, JA. Broad recognition of cytotoxic T-cell epitopes from the HIV-1 envelope protein with multiple class I histocompatibility molecules. J Immunol 1992;148:1657-67.

Takahashi H, Nakagawa Y, Pendleton CD, Houghten RA, Yokomuro K, Germain RN, Berzofsky JA. Analysis of CTL crossreactivity to an HIV-1 immunodominant determinant: elicitation of widely crossreactive CTL. In: Brown F, Chanock R, Ginsberg H, Lerner R, eds. Vaccines 92. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1992;69-74.

Takahashi H, Nakagawa Y, Pendleton CD, Houghten RA, Yokomuro K, Germain RN, Berzofsky JA. Induction of broadly crossreactive cytotoxic T cells recognizing an HIV-1 envelope determinant. Science 1992;255:333-36.

Patent Applications:

Berzofsky JA. Multideterminant peptide antigens that stimulate helper T lymphocyte response to HIV in a range of human subjects. Filed August 29, 1991. Application No. 07/751,998.

Berzofsky JA, Takahashi H, Germain RN. Method to induce cytotoxic T lymphocytes specific for a broad array of HIV-1 isolates using hybrid synthetic peptides. Filed September 18, 1991. Application No. 07/760,530.

Berzofsky, JA, Takeshita T, Shirai M, Pendleton CD, Kozlowski S, Margulies DH. Potent peptide for stimulation of cytotoxic T lypmhocytes specific for the HIV-1 envelope. Filed March 6, 1992. Application No. 07/847,311.

Shearer GM, Berzofsky JA, Clerici M. Test of HIV-specific T lymphocyte function that detects exposure to HIV antigens and possibly early HIV infection. Filed May 14, 1992. Application No. 07/882,078.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04024-5 MET

PERIOD COVERED: October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Analysis of Transacting Factors that Mediate Gene Expression

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

PI: Louis M. Staudt, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
Alex Dent, Ph.D.	IRTA Fellow	MET, NCI
Chi Ma, Ph.D.	Fogarty Visiting Associate	MET, NCI
Jaya Jagadeesh	Fogarty Visiting Associate	MET, NCI
Randall Maile	Special Volunteer	MET, NCI
Hon-Sum Ko, M.D.	Fogarty Visiting Associate	MET, NCI
Peggy Scherle, Ph.D.	IRTA Fellow	MET, NCI

COOPERATING UNITS (if any)
Robert Adelstein, Lab Molec Cardiol, NHLBI, Jonathan Yewdell, Lab Viral Disease NIAID
Kevin Holmes, Biol Resources Br, NIAID & Michael Lenardo, Lab Immunology, NIAID
Craig Venter, Receptor Biochemistry and Molecular Biology Section, NINDS

LAB/BRANCH
Metabolism Branch

SECTION

INSTITUTE AND LOCATION
DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	8	2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither "B" 100%
☐ (a1) Minors
☐ (a2) Interviews

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

Dr. Staudt's laboratory is taking three molecular approaches to understanding the development and function of lymphocytes. The first approach focuses on changes in the expression of transcription factors which take place when hematopoietic stem cells differentiate along the B cell lineage. In particular, Dr. Staudt has studied a novel population of mouse bone marrow progenitor cells which can differentiate, under appropriate conditions, into either B cells or myeloid cells. A variety of transcription factors studied were expressed in both the progenitor and differentiated cells. By contrast, two transcription factors, Oct-2 and LEF-1, were present at low or undetectable levels in the progenitor cells and are strongly induced upon differentiation to the lymphoid, but not the myeloid, lineage. Thus, Oct-2 and LEF-1 may play a role in commitment of a stem cell to the lymphoid lineage. The second approach involves the molecular cloning of novel lymphoid-restricted genes using subtractive hybridization techniques. One such gene, Ly-GDI, encodes a protein bearing striking homology to a regulator of the ras-like GTP-binding protein, rho, and may regulate events during lymphocyte activation. Another protein, JAW-1, has homology to the coiled-coil region of myosin and, surprisingly, resides in the endoplasmic reticulum. Both of these proteins reveal tissue-specific regulation of cellular processes that were previously thought to behave similarly in all cell types. The final approach involves the rapid and large-scale sequencing of cDNAs derived from subtracted and conventional cDNA libraries prepared from normal human lymphocytes or human lymphoid malignancies such as Burkitt's lymphoma and chronic lymphocytic leukemia.

Project Description

Major Findings:

Gene Expression During Lymphocyte Development

One molecular approach to an understanding of B lymphocyte development relies on the observation that each stage in B cell differentiation is accompanied by changes in gene transcription. A variety of transcription factors have recently been cloned which have been implicated in the expression of immunoglobulin genes in B cells. Some factors, such as the POU-domain protein Oct-2, are expressed in tumor cell lines derived from the B cell lineage but not in many other cell lines. Other factors, exemplified by the helix-loop-helix protein E47, are expressed in all cell lines tested thus far. Since all analysis of these transcription factors to date has been performed in cell lines, we have studied (in collaboration with Dr. Kevin Holmes) the expression of several factors in non-transformed hematopoietic cells. In particular, we have used an in vitro system in which non-transformed lymphoid/myeloid precursors derived from mouse bone marrow can be induced to differentiate, in different culture conditions, along either the B cell or myeloid lineages.

Using a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay, the levels of mRNA encoding a variety of transcription factors were measured in the lymphoid/myeloid precursor cells and in the B cell and myeloid cell cultures derived from them. Two factors, Oct-2 and the HMG-domain factor LEF-1, were expressed at low or undetectable levels in the precursors and were induced 50-200 fold upon differentiation to the B cell lineage but not the myeloid lineage. This observation argues strongly that Oct-2 and LEF-1 participate in commitment to the B cell lineage. Other factors, such as the Ets-domain proteins PU.1 and ets-1, were expressed in the precursors as well as in both B cells and myeloid cells suggesting that they are not involved in distinguishing these two cell types. One surprise was the observation that E47 was at very low levels in the precursors and was induced 50-100 fold upon differentiation to either the B cell or myeloid lineages. This result was not predicted from previous experiments with transformed cell lines and suggests that up-regulation of E47 is necessary to allow differentiation of the precursor cells. Thus, the differentiation of B cells from their uncommitted precursors involves the coordinate actions of a variety of transcription factors.

Isolation of Lymphoid-restricted cDNAs by Subtractive Hybridization

We have initiated a major new effort to isolate novel genes which control the differentiated functions of human B lymphocytes. One approach has involved the generation, by subtractive hybridization, of cDNA probes that are enriched in lymphoid-restricted cDNAs. Two such probes, prepared using cDNA from different human B lymphoma cell lines subtracted with mRNA from a human erythroleukemia cell line, were used to differentially screen a full length cDNA library prepared from a third human B lymphoma cell line. The majority of cDNAs which hybridized with both subtracted probes were lymphoid restricted in their expression and two novel genes were chosen for detailed analysis.

One lymphoid-restricted gene, JAW-1, showed an extended homology with the coiled-coil domain of skeletal muscle myosin. Using rabbit polyclonal anti-JAW-1 antibodies, we have shown (in collaboration with Dr. Jon Yewdell) that JAW-1 is localized in the endoplasmic reticulum (ER) which is surprising since previously studied ER proteins have invariably been expressed in all cell types. Biochemical studies have shown that JAW-1 associates with the ER by an unusual mechanism in which a hydrophobic domain located near its carboxy terminus is inserted into the ER membrane and the carboxy-terminal 30 amino acids are proteolytically cleaved. The coiled-coil domain of JAW-1 faces the cytoplasm where it potentially forms homodimers and/or heterodimers. Immunoprecipitation studies have revealed that a protein of approximately 200 kD associates with

JAW-1. A leading candidate for this associated protein is non-muscle myosin since antibodies to non-muscle myosin (obtained from Dr. Robert Adelstein) coprecipitate JAW-1. Our working hypothesis, therefore, is that JAW-1 tethers ER vesicles to the cytoskeleton allowing for vesicle movement.

Another novel lymphoid-restricted gene, Ly-GDI, bears striking homology to GDI, an inhibitor of GTP-GDP exchange for the ras-like protein, rho. Ly-GDI is expressed in spleen and thymus but not in other tissues which places it in marked contrast with other regulators of ras-like GTP binding proteins that are expressed in all cell types. Rabbit polyclonal antibodies to Ly-GDI have revealed that it is located diffusely in the cytoplasm. Ly-GDI coprecipitates a 23 kD GTP binding protein that is likely to be a member of the rho family. By analogy with GDI, we hypothesize that Ly-GDI binds to rho and keeps rho in its inactive, GDP-bound, state. The role of Ly-GDI in lymphocyte activation and proliferation is currently under investigation.

Large-scale Sequencing of cDNAs Derived From Normal and Neoplastic Human Lymphocytes

The advent of automated DNA sequencers and robotic workstations for the preparation of DNA sequencing reactions has made feasible the rapid sequencing of large numbers of cDNAs and thus the ability to generate a cDNA sequence profile of a given cell type. We are applying this approach to normal and neoplastic human lymphocytes in collaboration with the laboratory of Dr. Craig Venter. We have partially sequenced 200 cDNAs from a cDNA library that we prepared from the Burkitt's lymphoma cell line, BJAB, and have found that 70% of the cDNAs have not previously been identified. We have prepared several additional libraries for this effort including subtracted libraries enriched for human lymphocyte-restricted genes and conventional cDNA libraries from leukemic cells freshly isolated from patients with chronic lymphocytic leukemia (CLL).

Honors and Awards:

1991 Arthur S. Flemming Award

Publications:

Kang S-M, Tsang W, Doll S, Scherle P, Ko H-S, Tran A-C, Lenardo MJ, Staudt LM. Induction of the POU-Domain transcription factor Oct-2 during T cell activation by cognate antigen. 1992; Molecular and Cellular Biology, in press.

SUMMARY REPORT
EXPERIMENTAL IMMUNOLOGY BRANCH
October 1991 - September 1992

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

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 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 found that release from intra-thymically generated inhibitory CD4 signals results in increased TCR expression, dephosphorylation of TCR-zeta chains, and improved TCR signaling. Further, Dr. Singer's laboratory has found that the molecular basis for low TCR expression in developing $CD4^+CD8^+$ thymocytes is a high rate of degradation of newly synthesized TCR components, and that intra-thymically generated CD4 signals mediated by p56 lck regulate the TCR degradation rate in $CD4^+CD8^+$ thymocytes.

Dr. Alfred Singer's laboratory has also examined the intra-thymic differentiation of functionally and phenotypically distinct T cell subsets as well as their interaction with thymic epithelium (9273). Studies on thymocytes from genetically defective scid mice have suggested that TcR^+ 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. Phenotypic studies on developing thymocytes have identified two distinct, but inter-related subsets of thymocytes that express identically skewed TCR repertoires, namely $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes and $Ly6C^+$ thymocytes. $Ly6C^+$ thymocytes

were found to represent a readily identifiably subpopulation within each CD4/CD8 thymocyte subset; nevertheless, the $Ly6C^+$ thymocytes within each CD4/CD8 thymocyte subset expressed a distinctive TCR repertoire marked by overexpression of Vβ8 and expression of autoreactive TCR. Finally, Dr. Singer's laboratory found that thymocytes readily acquire surface CD4 and CD8 determinants from other thymocytes, demonstrating that caution is necessary in using low level CD4/CD8 expression to identify novel thymocyte subsets.

The process of negative selection, by which potentially self-reactive T cells are deleted during development, has been analyzed in the laboratory of Dr. Richard Hodes (9265). An analysis was carried out to determine 1) the extent of T cell receptor (TCR) Vβ deletions that occur in generation of the mature TCR repertoire, 2) the range of self determinants that play a role in these TCR deletions, and 3) the relationship of these "deleting ligands" to the strong alloantigens that mediate high frequency responses by mature T cell populations. Determination of TCR Vβ expression was carried out by mRNA quantitation as well as with monoclonal antibodies specific for individual Vβ products, in a large panel of inbred strains. Strain-specific deletions in 12 of the 22 Vβ products were detected and were shown to be related to the expression of multiple MHC and non-MHC self determinants. These findings indicate that maintenance of tolerance to a variety of self determinants results in substantial deletions in the available TCR Vβ repertoire (9265). The self determinants that function as ligands for Vβ-specific T cell deletions were shown generally to represent the products of non-MHC-encoded genes in association with MHC gene products. In several cases, a novel "genetic redundancy" was identified in the non-MHC ligands for Vβ deletion, such that any one of two or more unlinked genes was permissive for deletion. Ligands responsible for deletion of Vβ11- and Vβ12-expressing T cells were characterized and were shown to represent a previously uncharacterized Mls "superantigen" capable of inducing a strong response by allogeneic T cells. Thus, the set of Mls superantigens appears to be more extensive than was previously appreciated, and these antigens play a critical role as self determinants in shaping the TCR repertoire by negative selection. Mapping of the non-MHC genes contributing to Vβ-specific deleting ligands has in each case identified an endogenous mouse mammary tumor (MMTV) provirus associated with deletion.

The role of the thymus in TCR negative selection was analyzed in Dr. Hodes' laboratory by examining TCR Vβ expression in T cells which have matured in congenitally athymic nude mice (9265). It was found that deletions of Vβ3 and Vβ11 that normally occur in mice expressing appropriate non-MHC products in association with MHC class II determinants fail to occur in the $CD4^+$ and $CD8^+$ T cells of athymic mice, demonstrating that efficient negative selection is thymus-dependent.

Exogenous retroviruses were analyzed for their influences on T cell repertoire. Milk-borne MMTV induced Vβ14 deletion only in strains of mice bearing natural or transgenic I-E class II major histocompatibility complex (MHC) product. 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.

Although Vβ-specific negative selection mediated by endogenous superantigens provides 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. An analysis of the co-expression of specific Vα/Vβ pairs by individual 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. Additional evidence for the importance of Vα/Vβ 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 Vβ expression, plays a role in T cell specificity for endogenous mtv superantigen.

Dr Shaw's laboratory has been systematically analyzing heterogeneity among subsets of human T cells and the functional capacities of those subsets (9257). The concept that adhesion molecules often mark T cell subsets, which is based substantially on work from Dr. Shaw's laboratory, has been confirmed and extended by many aspects of his laboratory's studies this year. Continuing analysis has emphasized β1 and β7 integrins and CD31, because of their known importance in adhesion and adhesion regulation. His lab has identified a unique monoclonal antibody Act-1 and structurally characterized the molecule recognized as the $\alpha4\beta7$ integrin. Among CD4 cells $\alpha4\beta7$ is selectively expressed at high levels on unique subset of memory cells; indeed β1 and β7 are reciprocally regulated on CD4 memory cells. $\alpha4\beta7$-high CD4 cells are phenotypically unique among memory cells in several other respects and by multiple criteria are inferred to be gut homing T cells. Phenotypic analysis of gut lamina propria T cells (LPL) confirms many similarities between those cells and the $\alpha4\beta7$ circulating cells, but raises new complexities apparently due to partial in situ activation by the gut environment. Systematic three and four color analysis of human peripheral blood, lymphoid tissue and gut LPL are being conducted for these and other markers to deduce the potential functional properties of markers in lymphocyte homing. CD31 shows variation in expression of T cells in various secondary lymphoid tissue and nonlymphoid tissue, consistent with a role in regulating T cell migration.

The Cell Mediated Immunity Section 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; and c) patients with systemic lupus erythematosus (SLE) (9267). It was found that both HIV+ individuals and SLE patients exhibit a spectrum of TH functional defects which are predictive for disease progression and are associated with changes in the profiles of immunoregulatory cytokine production, including interleukins 2, 4, and 10. A significant number of HIV-exposed, seronegative individuals from high risk groups were found to exhibit in vitro TH function to synthetic peptides of HIV gp120. Studies in these at-risk groups and newborn infants to HIV+ mothers suggest that HIV-specific TH function is protective against HIV infection and/or progression to AIDS.

2. CELL BIOLOGY OF IMMUNE RESPONSES

The expression and function of cell adhesion molecules by B cells was analyzed by Dr. Hodes' laboratory (9266). IL5 induces B cell proliferation and immunoglobulin (Ig) secretion and results in appearance of a phenotypically novel B cell population which expresses high density of CD44 and low densities of B220 (CD45) and Ia. This B cell sub-population mediates nearly all of the proliferative and Ig secretory activity of IL5-activated B cells. In addition, the CD44 expressed by these cells mediates binding to the extracellular matrix material hyaluronic acid (HA), indicating a potential role for CD44 in regulating trafficking of activated B cells in vivo. The CD44 expressed on IL5-stimulated B cells migrates with a lower molecular weight than does CD44 expressed by control B cells, reflecting differential glycosylation. Other B cell activating stimuli such as LPS do not induce CD44-dependent HA-binding activity. However, LPS-activated B cells demonstrate CD44-dependent HA binding rapidly after exposure to a unique CD44-specific mAb, suggesting that distinct functional states of the CD44 molecule exist, perhaps reflecting differences in conformation or cytoskeletal association.

T cells at various stages of activation and differentiation are known to express different isoforms of cell surface CD45, reflecting in part the differential splicing of several variable exons. In contrast, B cells have generally been characterized as expressing a uniformly high molecular weight isoform of CD45. Analysis of resting and activated B cells demonstrated that activation-specific changes are induced in the expression of serologically detected CD45 epitopes. These changes can be correlated with changes detected by immunoprecipitation. In addition, a polymerase chain reaction (PCR) analysis of CD45 mRNA expression indicates that unique changes in variable exon splicing are induced by specific B cell activation stimuli.

In order to identify previously uncharacterized activation molecules expressed on lymphocytes, a series of mAb was generated by immunizing rats with activated mouse B cells. One of these mAb (GL7) reacts with a subpopulation of activated B cells, as well as with activated T cells. GL7 precipitates what appears to be a previously undescribed 29-31 KDa molecule from activated B cells. Another mAb generated in this fashion (GL1) reacts with activated B but not T cells. GL1 inhibits responses of $CD4^+$ T cells to activated B cells, suggesting that the target of GL1 may represent a costimulatory molecule for T cell activation.

Immunoglobulin (Ig) secretion was analyzed in a model system employing Ig μ/k 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.

Dr. Shaw's laboratory has been identifying and characterizing the functions of cell surface molecules which participate in T cell recognition and adhesion (9257). Studies are continuing on the family of molecules on T cells which have the capacity to trigger integrin-mediated adhesion, particularly CD31. CD31 has been shown to be a unique trigger molecule in: 1) sensitivity to bivalent cross-linking; 2) preferential induction of VLA-4 integrin function; and 3) expression on unique T cell subsets. Furthermore, perturbation of CD31 has multiple effects on T cell activation indicating a role for CD31 in activation of both T cells and monocytes. As noted above under section 1, a monoclonal antibody has been identified that is specific for the integrin $\alpha 4\beta 7$; the function of $\alpha 4\beta 7$ on lymphocytes has been probed using that mAb. $\alpha 4\beta 7$ is involved in binding to both fibronectin and VCAM-1; Act-1 blocks cell binding to the former and augments binding to the latter. Studies are continuing on the functional roles of multiple adhesion pathways in T cell interactions with endothelium. A new aspect of these studies is analysis of in vitro expanded TIL (tumor infiltrating lymphocytes). Phenotypic analysis demonstrates dramatic differences between the surface phenotype of these cells and that of normal circulating T cells. Studies of the molecular mechanisms for TIL binding to HUVEC cells reveal generally the same rules as have been observed with resting CD4 cells. The most striking difference is the fact that the integrins on TIL are already in a functionally activated state and show little further augmentation in binding when activated by various agents.

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.

In Dr. Hodes' laboratory, subpopulations of peripheral and thymic T cells were defined by flow cytometric analysis of CD45 isoform expression using mAb that detect CD45 exon-specific epitopes, including a novel exon C-specific mAb recently in this laboratory (9281). The exon-specific epitopes detected by these mAb were expressed in non-identical distributions in peripheral and thymic populations. In the thymus, those cells which stain most brightly with antibodies to CD45 exons B and C were found to constitute a unique population that express an intermediate level of TCR. TCR cross-linking with biotinylated anti-TCR mAb induced increased intracellular $[Ca^{++}]$ in mature peripheral T cells and in $CD4^{+}8^{-}$ and $CD4^{-}8^{+}$ thymocytes, and lesser responses in $CD4^{+}8^{+}$ thymocytes. Co-cross-linking of CD45 and TCR with biotinylated mAb resulted in profound inhibition of Ca^{++} responses by peripheral T cells, but had less pronounced effect on the responses of thymic T cells. These results suggest that the functional coupling of CD45 to the TCR may differ in T cell subsets.

Signal transduction pathways induced by endogenous superantigen stimulation of T cells were analyzed with both cloned and heterogeneous responding T cells. 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.

Adhesion molecules regulate T cell activation; studies from the laboratory of Dr. Shaw have helped establish this concept for human T cells (9257). In well-defined model systems of T cell activation, they are exploring which molecular pathways of adhesion facilitate T cell activation, the biochemical basis for the activation, and possible differences between different molecular pathways in the details of that activation. Recent studies have characterized changes in second message which may account for the costimulatory effect of integrin ligands. These studies demonstrate signal

transducing events resulting in prolonged phospholipase C (PLC) activation and PIP_2 hydrolysis, and a sustained increase in $[Ca^{2+}]_i$ level.

Studies in Dr. Weissman's laboratory have focused on the ζ subunit of the T cell antigen receptor (TCR) (9292). The ζ subunit of the TCR undergoes tyrosine phosphorylation in response to receptor engagement and is important in TCR-mediated signal transduction. As a consequence of tyrosine phosphorylation the reduced Mr of ζ increases from 16 to 21 kDa. The degree of this shift, coupled with a significant shift in pI, suggests that multiple residues are phosphorylated. To determine the particular tyrosine residues that are phosphorylated, mutagenesis of each of the intracellular tyrosines of ζ was carried out. This analysis revealed that each of the mutations was associated with a reproducible pattern of tyrosine phosphorylation. The Mr shifts seen with each of the mutants led to the conclusion that all of the 6 intracytoplasmic tyrosine residues of ζ were capable of being phosphorylated. This analysis suggested a clear heirarchy with the 4 most carboxy residues being most important to this process. Mutation of tyrosine 111 led to a profound dimunition in the level of phosphorylation. In addition, this analysis confirmed that the 21 kDa phosphorylated form of the receptor was generated by multiple phosphorylation events. Analysis of IL-2 production in response to receptor engagement was evaluated. No single tyrosine residue was essential for receptor-induced IL-2 production.

The molecular mechanisms responsible for the regulation of tyrosine phosphorylation of the TCR remain to be determined. To address this issue Dr. Weissman's laboratory has developed a permeabilized cell system to determine whether GTP binding proteins are involved in coupling TCR occupancy to transmembrane signalling (9292). Treatment of permeabilized cells with GTPγS did not stimulate ζ phosphorylation. When GTPγS was added to cells prior to receptor cross-linking, a significant increase in antibody-induced ζ phosphorylation was achieved. This GTPγS effect was dose-dependent and varied from 2-10 fold over antibody alone. Another non-hydrolyzable GTP analogue, GppNHp, resulted in the same effect. The specificity of this effect for GTP analogues was assessed by treatment of cells with non-hydrolyzable ATP analogues and with GDPβS None of these reagents resulted in any significant increase in antibody mediated ζ phosphorylation. These results strongly suggest that GTP binding proteins are involved in synergizing receptor-mediated tyrosine phosphorylation of ζ. Studies using pertussis and cholera toxins excluded a role for G_i or G_s in this process. The data from these studies suggest that G proteins play a synergistic role in enhancing phosphorylation induced by anti-receptor antibody. These are the first data to implicate G proteins as positive modulators of activation-induced tyrosine phosphorylation.

In the course of the analysis of human, rat, and murine ζ immunoblots, Dr. Weissman's laboratory noted previously unappreciated degrees of

heterogeneity (9296). Based on the finding of multiple species that were immunoreactive with anti-ζ reagents and which differed from each other by 8 kDa, it was postulated that ζ might undergo ubiquitination. To test this possibility, immunoprecipitates from 2B4 cells were resolved on two dimensional gels. Immunoblotting was carried out with monoclonal anti-ubiquitin reagents, which revealed a ladder of activation-induced species that co-migrated with the activation-induced immunoreactive forms of ζ. This finding indicated that ζ was ubiquitinated. Subsequently, Dr. Weissman's laboratory found that CD3 δ is also ubiquitinated. Further analysis has led to the conclusion that phosphorylated ζ can also serve as a substrate for ubiquitination. These ubiquitinated subunits are not isolated intermediates in a pathway to degradation but are components of apparently intact TCRs. Whether these receptors are in fact slated for degradation remains to be determined. Additional studies have determined that this alteration occurs in normal human cells and human T cell tumor lines. Kinetic studies have demonstrated ubiquitinated forms within 5 minutes of stimulation with their persistence for at least 2 hours. This process occurs in all T cell types that have been analyzed.

4. STRUCTURE, REGULATION AND FUNCTION OF GENES AND PROTEINS INVOLVED IN IMMUNE RESPONSES

The laboratory of Dr. 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. 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 have been investigated.

Dr. Segal's group has made a genetic construct of an sFv of a murine T cell receptor, expressed it in bacteria, and refolded it to produce sFv protein in 10-100 mg amounts (9289). The resultant protein is monomeric in aqueous solution and contains three epitopes that are present on the parental TCR for which we have mAbs. These data show that isolated variable domains of the TCR can form a well defined structure with immunological properties of the native molecule, and in this regard, behaves like immunoglobulin. This

protein will be used to study the specificity and structure of the T cell receptor binding site.

Genomic clones encoding the human ζ gene were characterized in Dr. Weissman's laboratory (9291). Analysis of the 5' region of the gene revealed the absence of classic and non-classic CAAT or TATA boxes. Using ribonuclease protection and primer extension a number of sites of transcription initiation were found distributed over 115 bases, with a heavily utilized transcription initiation site 66 bases from the translation initiating ATG. Through hybridization of the human ζ cDNA to genomic DNA from unrelated donors, the ζ gene was determined to contain a variable number tandem repeat (VNTR). The ζ VNTR was localized to the exon 5/intron 5 boundary and found to be a unique 36 base pair repeat. This VNTR allowed Dr. Weissman's laboratory to determine the position of the ζ gene on human chromosome 1q. They demonstrated that ζ is positioned in a region where a cluster of Fcγ receptors had been localized by in-situ hybridization. Dr. Weissman's laboratory determined that ζ and the FcγRII-FcγRIII gene cluster are linked, within 10 centimorgans, on human chromosome 1q.

The η subunit of the murine TCR is an alternative splice of the murine ζ gene (9291). Analysis of human genomic clones isolated in Dr. Weissman's laboratory revealed the presence of a region homologous to that described as the murine η exon. This region is highly conserved on a nucleotide level (77%). However multiple insertional and deletional mutations result in frameshifts and therefore marked differences in the deduced primary amino acid sequences of these regions. Ribonuclease protection was utilized to determine whether human η transcripts exist. These studies revealed that the η exon is transcribed and spliced at extremely low levels in human T cells. The lack of conservation in the η region, coupled with its extremely low level of expression, led to an evaluation of this region in other mammals. This analysis revealed a high degree of conservation of the putative intron/exon boundary as well as the first 21 bases of the η exon. Hence the first 7 amino acids specific to η are conserved. Beyond this region a high degree of frameshifting was observed with no subsequent conservation of deduced amino acid sequence. There were also highly conserved blocks of nucleotide sequence in which no frame shifting occurred. Thus the η exon is exceptional, as there is conservation of nucleic acid sequence but not of deduced amino acid sequence.

5. LYMPHOCYTE EFFECTOR FUNCTION

In studies on the mechanism of lymphocyte-mediated cytotoxicity, P. Henkart's laboratory has established the role of cytotoxic lymphocyte granzymes in triggering "apoptotic" damage to target cells, including DNA fragmentation (9251). Their approach was transfection of a mast cell tumor line with combinations of the lymphocyte granule components cytolysin and granzyme A. Transfectants expressing only the membrane pore-forming

cytolysin killed targets without DNA breakdown, while transfectants expressing only granzyme A were not cytotoxic. Transfectants expressing both showed tumor target cytotoxicity with accompanying DNA breakdown. They furthermore showed that injection of several different well characterized proteases into tumor cells triggers apoptotic cell death, with DNA breakdown preceding lysis, membrane blebbing and cell shrinkage. These data further support the granule exocytosis model for lymphocyte-mediated cytotoxicity.

6. DEVELOPMENTAL BIOLOGY

Dr. Kuehn's laboratory has used insertional mutagenesis to identify genes that have important roles in mouse embryogenesis (9297). Mouse embryonic stem cells, infected with a retroviral vector in order to induce insertional mutations, have been injected into embryos, resulting in transgenic mice carrying multiple independent mutations in the germ line. As a result of analyzing two transgenic strains, Dr. Kuehn's laboratory identified 27 proviral insertions, five of which were associated with recessive mutations that disrupt embryonic development. Further analysis of one of the mutant genes has revealed that it is a lethal recessive that causes early death of the embryo associated with hyperplasia of the embryonic and extra embryonic ectoderm. The gene, named hyperplastic ectoderm (hec), is located on chromosome 10 and is being cloned for molecular analysis.

Techniques have been developed in Dr. Kuehn's laboratory for improving the efficiency of following the inheritance patterns of large numbers of proviruses intentionally inserted into the germ lines of transgenic mice (9298). Eighteen different retroviral vectors have been created by inserting different fragments of phage lambda DNA into a basic retroviral vector. Because each vector has a unique molecular tag, its insertion site in the germ line of a transgenic mouse strain can be detected among as many as 360 other retroviral insertions. This detection scheme represents a 20-40 fold increase in the number of insertions that can be studied in each transgenic mouse strain.

7. TRANSPLANTATION BIOLOGY

Studies in Dr. Alfred Singer's laboratory have attempted to apply our understanding of the cellular mechanisms involved in in vitro anti-MHC responses to in vivo transplantation responses (9275). In studying skin allograft rejection, Dr. Singer's laboratory has identified the phenotype, specificity, and interaction capabilities of the T cells able to initiate and effect in vivo rejection responses. They found that in vivo exposure of effector cells to skin allografts under conditions in which T-helper cells were not activated resulted in the inactivation of the effector cells and longterm retention of the skin allograft. In addition, Dr. Singer's laboratory found that rejection across a class I MHC barrier could occur in mice depleted of $CD8^+$ T cells by in vivo administration of anti-CD8 mAb, but

that the in vivo effector cells were a novel population of anti-CD8 resistant $CD8^+$ T cells that had down-modulated their CD8 surface expression and were highly resistant to anti-CD8 blockade of their cytolytic function. In addition, Dr. Singer's laboratory demonstrated that rejection of skin allografts across a class II MHC barrier requires the production of endogenous IFNγ, presumably to induce class II expression on all the cells of the graft and make them recognizable by class II allospecific effector cells. Finally, Dr. Singer's laboratory has assessed the cellular mechanisms mediating the rejection of fetal pancreas and islet cell allografts.

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. Because host CTL in isolation could reject donor marrow grafts, effects on engraftment by cell populations able to suppress host CTL responses, and the administration of anti-CD3 monoclonal antibody in vivo, which by previous work had been shown to suppress CTL function, were studied. Cells with a specific type of suppressor activity, termed veto cells, which might suppress host rejection responses, have been reported to be present in marrow. 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. In further studies of engraftment of T cell depleted allogeneic marrow, host mice were treated with anti-CD3 monoclonal antibody. The injection of anti-CD3 monoclonal antibody with the donor marrow resulted in extensive allogeneic chimerism. Incubation of T cell depleted allogeneic marrow with the supernatant of spleen cells incubated with anti-CD3 antibody in vitro also resulted in enhancement of engraftment in the presence of, but not in the absence of, host T cell suppression. Therefore, the enhancement of marrow engraftment by in vivo administration of anti-CD3 monoclonal antibody is due to both suppression of host T cell function and the presence of growth factors. Identification of specific factors which are present in the supernatants of spleen cells exposed to anti-CD3 monoclonal antibody, and which promote engraftment of T cell depleted, MHC-mismatched marrow, indicates that multiple factors are involved.

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. Preclinical studies demonstrated that functional T cell populations are generated in animals receiving T cell depleted autologous marrow. 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 number or 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 residual T cells in the infused marrow play a central role in the generation of subsequent T cell populations in the recipient. This possibility has been confirmed in murine studies in which three T cell progenitor pools have been identified which contribute to final T cell repopulation following marrow transplantation. The functional capacities of regenerated T cell populations following T cell depleted marrow transplantation is also of interest. The human T helper cell response to xenogenic MHC encoded antigens expressed by stimulating murine cell populations has been studied and found to be of special use in the assessment of human T helper cell function in that this primary response requires reprocessing of the stimulating murine antigens and presentation in association with human Class II gene products. 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. 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.

The relationship of transplantation tolerance to TCR expression was studied in Dr. Hodes' laboratory using mixed xenogenic radiation bone marrow chimeras (9265). The reconstitution of irradiated mice with innocula containing mixtures of mouse and rat bone marrow cells resulted in stable chimerism of mouse and rat cells which were mutually tolerant as reflected in vivo and in vitro. This tolerance was accompanied by deletions of mouse T cells expressing specific Vβ products in a fashion that was specific for

the strain of rat employed. Thus xenogeneic tolerance was accompanied by was potentially mediated by specific deletion of mouse T cells.

The Cell Mediated Immunity Section is investigating the mechanisms of human solid organ allograft rejection (9264). It was found that renal allograft rejection is mediated only by recipient anti-donor CD4+ T helper cell responses that are activated by alloantigen, processed and presented on recipient antigen-presenting cells (APC). In contrast, liver allografts appear to be rejected both by processed alloantigens and alloantigens presented directly on donor APC. The rejection of renal allografts could be predicted by an in vitro T cell test developed by the laboratory.

8. TUMOR IMMUNOLOGY

In Dr. Hodes' laboratory, analysis of TCR Vβ expression has been carried out to characterize the in vivo response of mice to syngeneic tumors (9265). Freshly isolated tumor-infiltrating lymphocytes (TIL) as well as in vitro lines derived from TIL were characterized. The expression of TCR Vβ products in freshly isolated TIL was non-random but nevertheless was highly heterogeneous. Long-term TIL lines were frequently oligoclonal, but with no consistent relationship between TCR Vβ usage and T cell specificity. The response to syngeneic tumors in this system therefore does not show any detectable predominance in TCR Vβ expression.

A mouse model for retargeting the immune system against syngeneic mammary tumors has been studied in the laboratories of Drs. Segal and Wunderlich (9250,9254). The model utilizes tumors, both primary and passaged, induced by the vertically transmitted mammary tumor virus. The specificities of cytotoxic T lymphocytes from normal donors have been retargeted with bispecific antibodies, so that the T cells react against a virus-related envelope protein selectively expressed on the surface of the tumor cells. The bispecific antibody used in this effort reacts both with a CD3 component of the T-cell receptor complex and with a gp52 antigen on the tumor cells. The investigators found 1) that retargeted cytotoxic mouse splenocytes lyse both passaged and freshly explanted primary tumor cells in vitro and 2) that they block the growth of syngeneic tumor cells in subcutaneous tumor neutralization (Winn) assays. There are two requirements for successful retargeting in these tests. First, the effector cells need to be preactivated, and second the bispecific antibodies need to be crosslinked rather than physically mixed. The investigators also found that targetable cytotoxic T cells can be effectively activated either in vitro or in vivo; however, used alone without bispecific antibodies, the activated T-cells did not affect tumor growth. Similarly, bispecific antibodies used alone fail to affect tumor growth.

9. FLOW CYTOMETRY

The EIB flow cytometry laboratory (9255) continues to support multiple investigations which involve quantitative, single cell, multiparameter immunofluorescence analysis of cells prepared from a variety of tissues and species, as well as a spectrum of in vitro cultured cells (9268, 9273, 9265, 9257, 9266, 9281, 9275, 9287, 9288).

During the past year, the EIB flow cytometry laboratory (9255) has implemented use of new flow cytometry instrumentation for research support. This implementation enabled identification of multiple necessary modifications to instrumentation, computer hardware, and computer software. Modifications have been designed and implemented to enhance throughput, functionality and flexibility of flow cytometry instrumentation and software.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

PI:	John Wunderlich	Senior Investigator	EIB, NCI
Others:	David Segal	Section Chief	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	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.)*

Cytolytic mouse T cells were successfully retargeted with bispecific antibodies, so that they lysed, in vitro, syngeneic breast cancer cells induced by the mammary tumor virus. The bispecific antibody reacted with a triggering site on the surface of T cells - a CD3 component within the T-cell receptor complex,and with a virus-related antigen expressed on the surface of the tumor cells. Two requirements for the antitumor activity were that 1) the T cells had to be preactivated, and 2) the antibodies comprising the bispecific reagent had to be crosslinked and not simply mixed together. We preactivated the T cells by culturing splenocytes from normal donors with irradiated allogeneic cells, bacterial lipopolysaccharide, and recombinant IL-2.

Preactivated mouse spleen cells also blocked the growth of syngeneic breast cancer cells in tumor neutralization (Winn) assays in vivo, if retargeted against the tumor cells with the bispecific antibodies.

Project Description

Major Findings:

The focus of this laboratory has been on antitumor cytotoxic effector cells and factors that influence their generation. This year we worked on establishing a mouse model for retargeting cytotoxic T lymphocytes with bispecific antibodies, so that the T cells will react against syngeneic breast cancer cells induced by the mammary tumor virus.

Retargeting T lymphocytes against tumor cells.

Two fundamental requirements of cytotoxic T cells are 1) that they express receptors capable of triggering the release of cytotoxic molecules following ligand binding and 2) that they be sufficiently activated to produce and, following triggering, release the effector molecules that mediate cytotoxicity. With bispecific antibodies we have succeeded in providing the T cell receptors for recognizing tumor cells, as described in previous progress reports.

This year we have focused our efforts on 1) activating the T cells and 2) retargeting them with bispecific antibodies so that they react against breast cancer cells in a mouse model.

Breast cancer, induced by the murine mammary tumor virus (MTV), appears spontaneously in more than 75% of females from some strains of mice, such as MTV-positive C3H. Also, a variety of mammary tumor cell lines are available, as are monoclonal antibodies that react with MTV components expressed on the cell-surface of mammary tumor cells.

Earlier, we found that mouse cytolytic T cells, irrespective of their native specificities, can be retargeted to react selectively with mouse breast cancer cells induced by the mouse mammary tumor virus. Retargeting was achieved with bispecific antibodies that recognized the T-cell receptor and the viral envelope glycoprotein, gp52. Gp52 is expressed on the surface of MTV-induced tumor cells.

We found using flow cytometry that gp52 was expressed on most (perhaps all) MTV-induced primary tumor cells but not on splenocytes, with the exception of about 5% of cells that were probably a subpopulation of B lymphocytes. To disperse the tumor cells, we used a combination of collagenase, hyaluronidase, and DNAse that did not appear to affect the expression of cell-surface gp52. We also found that splenic cytolytic T cells, nonspecifically preactivated *in vitro* by high levels of recombinant IL-2, lysed MTV-induced primary tumor cells when retargeted against gp52 with bispecific antibodies. The retargeted cytolytic T cells did not affect splenocytes from tumor-bearing mice or tumor cells that did not express gp52.

This year we were able to address three issues that affect the retargeting of T cells against MTV-induced breast cancer cells.

First, how can cytolytic T cells be polyclonally activated without using high doses of recombinant IL-2? The need for high doses of IL-2 restricts the mouse strains available to us in which we can minimize potential problems with activated NK cells, and these available mouse strains do not include those with high incidences of spontaneous, MTV-induced breast cancer. Moreover, high doses of IL-2 cannot be used in vivo without serious toxic side effects.

To address this issue, we have tested the hypothesis that substantial activation of targetable cytolytic T cells should occur with allostimulation supplemented by low levels of recombinant IL-2 and bacterial lipopolysaccharide (LPS). Conceptually, the alloantigens should provide the primary activation signal to T cells and the IL-2 and LPS should stimulate helper T-cells and accessory cells, both of which support the generation of targetable cytotoxic T cells by providing costimulatory signals.

Indeed, optimal levels of targetable cytolytic T-cell activity were generated in vitro by culturing mouse splenocytes from normal adult donors with irradiated allogeneic cells, relatively low doses of recombinant IL-2 (10 U/ml), and LPS. Less activity developed when splenocytes were cultured with the individual agents or with paired combinations. Less activity also occurred when splenocytes were cultured with anti-CD3 or staphylococcal enterotoxin, alone or combined with IL-2 and LPS. Both anti-CD3 and staphylococcal enterotoxin are polyclonal T-cell activating agents.

In preliminary tests we have also been able to activate targetable cytolytic T-cells in vivo by injecting mice with a combination of allogeneic cells, LPS, and relatively low doses of recombinant IL-2. Targetable T cells were detected by using in vitro assays. By contrast, splenic T cells freshly explanted from normal adult mice were inactive in targeted cytolysis tests.

The second issue that we investigated concerns whether circulating gp52 will block cytotoxic T cells retargeted with bispecific antibodies that react with gp52. Earlier reports from other laboratories described circulating levels of about 750 ng/ml MTV gp52 in mice bearing spontaneous MTV-induced breast cancers. Will this level of cell-free gp52 occupy the anti-gp52 arm of the bispecific antibody to such an extent that the antibody will not be free to retarget cytotoxic T cells against $gp52^+$ tumor cells? To test this issue in vitro, we added purified MTV virus to retargeted cytolytic T cells and ^{51}Cr-labeled mammary carcinoma target cells. We found that cytolysis of $gp52^+$ tumor cells, caused by retargeted T cells, was not inhibited by purified MTV virus with a gp52 level of about 10 ug/ml and that the virus did not trigger the release of cytolytic factors. The same concentration of virus specifically caused greater

than 50% inhibition of complement-dependent tumor-cell lysis mediated by the concentration of bispecific antibody that was used for retargeting cytolytic T cells. These results indicate that cell-free levels of gp52 circulating in tumor-bearing mice will not block retargeted T cells.

The last issue that we investigated concerns whether cytotoxic T cells can be retargeted to react in tumor neutralization (Winn) assays against MTV-induced breast cancer cells. To address this question, we mixed splenocytes from BALB/c mice with the anti-CD3 x anti-gp52 bispecific antibody and 64PT tumor cells. These cells are an MTV-induced BALE/c mammary tumor line that produces MTV virus and expresses cell-surface gp52. The mixture was promptly injected subcutaneously into normal BALB/c mice.

With lymphoid to tumor cell ratios of 40 to 1 in one experiment and 10 to 1 in another, only 1 out of 15 mice from the two experiments developed tumors after 4 weeks.

By contrast, tumor growth was apparent in most of the control mice within 4 weeks, and in all cases tumors continued to grow until mice were sacrificed when the tumor diameter reached 2 cm. There were no spontaneous tumor regressions. Eighty percent or more of mice in each of the control groups developed tumors. These control groups included mice injected with tumor cells only, tumor cells with bispecific antibody alone, tumor cells with lymphocytes alone, and tumor cells with lymphocytes and antibodies that had been mixed rather than chemically crosslinked.

In the tumor neutralization assays described above, there were two requirements for optimal prevention of tumor growth by retargeted T cells. First, the T cells had to be preactivated. Freshly explanted, normal splenocytes mixed with bispecific antibodies were ineffective. We preactivated the T cells by culturing splenocytes for 5 days with a combination of irradiated allogeneic cells, IL-2, and bacterial lipopolysaccharide. Second, the bispecific antibodies had to be crosslinked; as described above, a mixture of separate antibodies was not effective. Previously, we demonstrated in other tumor models that the bispecific antibody must be directed against a triggering site on the T-cell, and that mere promotion of conjugation between the cytotoxic cells and tumor cells is inadequate.

Proposed Course

Retargeting mouse cytotoxic cells toward syngeneic breast cancer. We have two basic goals for this project. First, we plan to extend our understanding of how retargeted T cells act in vivo in mice that bear syngeneic passaged or primary breast cancers induced by the mammary tumor virus. Second, we want to determine if retargeted T cells can eliminate the established tumors in vivo. To these ends, we are planning to determine (1) the cellular basis for anti-

tumor activity of retargeted T cells against established tumors and the need for T cell activation, (2) how long retargeted T cells remain functional in vivo, (3) whether the retargeted T cells or bispecific antibody will home to a distant tumor site, (4) if T cells with genetically engineered receptors for tumor cell recognition are more effective than those bearing bispecific antibodies, and (5) if retargeted T cells will block tumor growth in situ. A detailed description of these plans is available in the 1992 report to the Board of Scientific Counselors.

Publications:

Wunderlich JR, Hodes RJ. Principles of tumor immunity: biology of cellular immune responses. In DeVita Jr., V.T., Hellman, S., and Rosenberg, S.A. Biologic therapy of cancer: principles and practice. Lippincott, Philadelphia, 1991, Chapter 1, pp 3-21.

Winkler DF, Myers KR, Hochstein HD, Ulrich JT, Wunderlich JR. Bacterial lipopolysaccharide acts synergistically with selected macromolecular polyanions to induce MHC-nonrestricted cytotoxic cells. Immunobiology 1991;182:216-233.

Segal DM, Qian J-h, Andrew SM, Titus JA, Mezzanzanica D, Garrido MA, Wunderlich JR. Cytokine release by peripheral blood lymphocytes targeted with bispecific antibodies, and its role in blocking tumor growth. Annal NY Acad Sci 1991;636:288-294.

Mezzanzanica D, Garrido MA, Neblock DS, Andrew SM, Zurawski Jr VR, Segal DM, Wunderlich JR. Human T-lymphocytes targeted against an established human ovarian carcinoma with a bispecific $F(ab')_2$ antibody prolong host survival in a murine xenograft model. Cancer Research 1991;51:5716-5721.

Wunderlich JR, Mezzanzanica D, Garrido MA, Neblock DS, Daddona PE, Andrew SM, Zurawski Jr VR, Canevari S, Colnaghi MI, Segal DM. Bispecific antibodies and retargeted cellular cytotoxicity: novel approaches to cancer therapy. Int J Clin Lab Res, in press.

Segal DM, Qian J-h, Mezzanzanica D, Garrido MA, Titus JA, Andrew SM, George AJT, Jost CR, Perez P, Wunderlich JR. Targeting of anti-tumor responses with bispecific antibodies. Mol Immunol, in press.

Meadows G, Wallendal M, Kosugi A, Wunderlich JR, Singer D. Ethanol induces marked changes in lymphocyte populations and natural killer cell activity in mice. Alcoholism: Clin Exptl Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09251-20 EIB

PERIOD COVERED: October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Target cell damage by immune mechanisms

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

PI:	P.A. Henkart	Section Chief	EIB, NCI
Others:	J. Shiver	Staff Fellow	EIB, NCI
	H. Nakamura	Visiting Fellow	EIB, NCI
	H. Park	Biologist	EIB, NCI
	M. Williams	IRTA 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 MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In order to test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the cytotoxic activity of the rat mucosal mast cell tumor line RBL after transfection with genes for cytotoxic lymphocyte granule components. In particular we have sought to test whether the granule serine proteases known as granzymes play a role in target DNA degradation accompanying cytotoxicity. We have constructed double and single RBL transfectants expressing cytolysin (cy) and the granule serine protease granzyme A (gza). RBL-cy transfectants show potent killing on red blood cell targets, and moderate cytotoxicity on tumor targets. However, DNA degradation in the latter is negligible. While RBL-gza transfectants express gza at levels comparable to cloned CTL and secrete it in response to IgER cross-linking, they have no cytotoxic activity detectable. RBL-cy-gza transfectants showing good expression of both these granule components showed cytolytic activity comparable to RBL-cy on both RBC and tumor targets. Target DNA breakdown by these double transfectants was clearly positive, with dose response curves parallel to lysis but somewhat less efficient. Agarose gels of labelled target DNA showed the ladder pattern characteristic of internucleosomal cleavage. These results clearly show that granzyme A can play a role in target DNA breakdown. As a direct test of the ability of proteases to induce cytotoxicity when introduced into the cytoplasm of a target cell, we have "injected" various proteases into tumor cells using osmotic lysis of pinosomes. The endoproteases trypsin, chymotrypsin, proteinase K, and papain were all found to lyse lymphoma cells in a dose dependent manner, as measured by ^{51}Cr release; this measure of death was preceded by DNA release. Microscopically, these cells were shown to undergo a marked membrane blebbing and shrinking, characteristic of "apoptosis".

Project Description

Major Findings:

In order to test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the cytotoxic activity of RBL cells transfected with genes for cytotoxic lymphocyte granule components. RBL, a rat mucosal mast cell tumor line which degranulates in response to cross-linking its IgE Fc receptor is not cytotoxic but aquires a potent lytic activity against IgE coated red cells when transfected with the mouse cytolysin (cy) gene. This potency is greater than that of cloned CTL with this target. Using tumor target cells, the RBL-cy give good cytotoxicity, although still 10-100X less than cloned CTL. When target DNA degradation accompanying lysis was examined with RBL-cy effector cells, it was not detectable, in striking contrast to that induced by CTL in the same experiment. Thus the cell-delivered cytolysin mimics the effects of purified cytolysin added to the medium. We have constructed double and single RBL transfectants expressing cytolysin and the granule serine protease granzyme A (gza). These transfectants express granzyme A at levels comparable to cloned CTL, target it to granules, and secrete it when the IgER is cross linked. RBL-gza show no cytotoxic activity, while RBL-cy-gza transfectants showing good expression of both these granule components have cytolytic activity comparable to RBL-cy on both RBC and tumor targets. When DNA breakdown in tumor targets was examined, it was clearly observed with RBL-cy-gza effectors, although not as potently as with CTL effectors. These preliminary results support our earlier evidence suggesting that granzyme A triggers target DNA breakdown after gaining access to the target cell cytoplasm. As a direct test of the ability of proteases to induce cytotoxicity when introduced into the cytoplasm of a target cell, we have "injected" various proteases into tumor cells using osmotic lysis of pinosomes. The endoproteases trypsin, chymotrypsin, proteinase K, and papain were all found to lyse lymphoma cells in a dose dependent manner, as measured by ^{51}Cr release; this measure of death was preceded by DNA release. Microscopically, these cells were shown to undergo a marked membrane blebbing and shrinking, characteristic of "apoptosis". The exoprotease carboxypeptidase had no effect when similarly injected.

Proposed course:

We have engineered the granzyme B gene into the SRα expression vector and set up RBL transfections using all combinations of cytolysin, granzyme A and granzyme B. We are particularly interested to see if the triple tranfectants have cytolytic activity on tumor targets comparable t o CTL. We will also be interested to see if granzyme B causes target nuclear DNA breakdown in combination with cytolysin alone. EM studies of the morphology of target cells injured by these transfectants will be carried out by collaboration. Cytotoxicity by protease injection is being studied further to elucidate its mechanism. The effect of various drugs on this cytotoxicity is being examined. We will attempt to define the physiologically relevant substrate which leads to lysis and to DNA breakdown.

Publications:

Kuta AE, Bashford L, Pasternack CA, Reynolds CW, Henkart PA. Partial characterization of nonlytic cytolylsin-RBC intermediates and their formation with tumor cells. Mol.Immunol. 1991;28:1263-1270.

Hiruma K, Nakamura H, Henkart PA, Gress RE. Clonal deletion of postthymic T cells: Veto cells kill precursor cytotoxic T lymphocytes. J.Exp.Med. 1992;175:863.

Shiver JW, Henkart PA. Cytolytic activity of RBL cells transfected with the cytolysin/perforin gene. In: NK Cell-Mediated Cytotoxicity: Receptors, Signalling, and Mechanisms, edited by Herberman, R.B. and Lotzova, E. Miami: CRC Press, 1991

Henkart PA, Hayes MP, Shiver JW. The granule exocytosis model for lymphocyte cytotoxicity and its relevance to target cell DNA breakdown. In: Cytotoxic cells: Recognition, effector function, generation and methods, edited by Sitkovsky, M.V. and Henkart, P.A. Boston: Birkhauser, 1992.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09254-17 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

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

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

PI:	D. M. Segal	Section Chief	EIB, NCI
Others:	D. Mezzanzanica	Guest Researcher	EIB, NCI
	S. Andrew	Visiting Fellow	EIB, NCI
	M. Mareno	Special Volunteer	EIB, NCI
	J. Wunderlich	Senior Investigator	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION
Immune Targeting Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

1. Human PBL that are targeted with bispecific antibodies exhibit an anti-tumor activity mediated by cytokines secreted into the medium as a result of T cell receptor crosslinking. This activity is different from targeted cytolysis as measured by ^{51}Cr release in that it leads to the eradication of both target and bystander tumor cells. Two cytokines involved in blocking tumor growth are TNF-α and IFN-γ. Induced cytokine release may be a major means by which targeted lymphocytes block tumor growth in vivo.

2. Targeted human PBL were tested for the ability to block the growth of established human ovarian carcinoma in nude mice. When given 4 days after tumor, PBL coated with anti-CD3 containing bispecific antibodies greatly decreased the number of mice bearing observable tumor at day 15, and at least doubled the long term survival times of tumor bearing mice.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

Project Description

Major Findings

Targeted tumor growth inhibition.

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. We have previously found that targeted human T cells exhibit a potent anti-tumor activity when injected subcutaneously with tumor cells in nude mice. Interestingly, however, we found that one subset of cells, $CD8^-$ T cells, was inactive in a 4 hour lysis assay, but was able to block subcutaneous tumor growth in mice. This suggested that targeted effector cells might mediate an anti-tumor activity different from lysis as measured in our standard assay. In order to study this phenomenon in greater detail, we established an in vitro tumor growth inhibition assay, in which we followed the effects of targeted lymphocytes on tumor growth over a 6-10 day period.

By using a combination of assays that measure ^{51}Cr-release over 4 hr, in vitro tumor growth inhibiton over 6-10 days in culture, or tumor neutralization in nude mice, we compared the mechanisms by which human PBL targeted with bispecific antibodies either lyse tumor cells or block their growth in culture or in mice. We found that targeted, resting PBL and $CD8^-$ T cells were unable to mediate lysis, but were able to block tumor growth in vitro and in mice. Moreover, targeted PBL were unable to lyse bystander cells but were able to block the growth of bystander tumor cells in culture and in a subcutaneous environment in nude mice. Supernatants from cultures of targeted PBL, or from PBL grown in the presence of immobilized anti-CD3 blocked the growth of tumor cells in the absence of added effector cells, and antibodies against TNF-α and IFN-γ reversed the inhibition of tumor growth, but had no effect upon cytolysis mediated by targeted PBL. Thus we have shown that targeted human PBL mediate two different anti-tumor activities; lysis, which occurs rapidly and requires the direct attachment of the target cell to the cytotoxic cell, and tumor growth inhibition, which is mediated by cytokines, including TNF-α and IFN-γ, released into the medium as a result of receptor crosslinking. The inhibition of bystander tumor growth in mice by targeted PBL indicates that factor release is important in blocking tumor growth in vivo. Targeted factor release therefore provides a mechanism by which targeted PBL could block the growth of tumor cells in vivo that were not bound by the effector cells, but which were located in the vicinity of tumor cells that were bound.

Targeted cytotoxicity in vivo; xenogeneic model

We have established a system to test whether targeted cytotoxic cells can eradicate an established tumor. Nude mice were given intraperitoneal injections of cells from the human ovarian adenocarcinoma line, OVCAR3, on day 0, and the mice were treated with human PBL with or without bispecific antibodies on day 4. The intraperitoneal growth of OVCAR-3 tumor cells was well-established on day 4: the average number of tumor cells recovered by peritoneal lavage was twice the

number originally injected. By histological examination on day 4 we were also able to detect implanted tumor cells within the pancreas and mesenteric lymph nodes. In this system both the tumor and the treatment are primarily in the peritoneum, thereby minimizing problems of delivering the targeted cytotoxic cells to the tumor.

In short term assays, where mice were sacrificed after 15 days and peritoneal lavages were tested for tumor cells, we found that targeted PBL were highly effective at eradicating tumor growth. Treating tumor-bearing mice with preactivated T cells, targeted against the tumor with $F(ab')_2$ bispecific antibodies, resulted in 80% of the mice having little or no detectable tumor in peritoneal lavage fluid collected 11 days after treatment. In controls where mice were given PBL alone, or PBL with either parental antibody (not crosslinked), only 6 to 20% of tumor-bearing mice were tumor free at this time. In long-term experiments, the mean survival time of tumor-bearing mice was greatly enhanced by treating them with the targeted lymphocytes instead of lymphocytes alone or lymphocytes with either parental antibody. Thus the mean survival time of tumor-bearing mice treated with PBL and bispecific $F(ab')_2$ was 104 days, which was 3.5 times that of untreated mice, and twice that of mice given PBL alone or PBL with either parental antibody. These results indicate that treating ovarian cancer patients with targeted T cells could prove beneficial, thus providing an animal model rationale for clinical studies using bispecific antibodies to treat ovarian cancer patients, now commencing in The Netherlands and in Italy.

Targeted cytotoxicity in vivo; syngeneic model

Our previous studies of targeted cytotoxicity in vivo have suffered from lack of a syngeneic model, i.e. a murine tumor growing in a syngeneic mouse strain, treated with a bispecific antibody specific for the murine tumor and murine TcR. We now believe we have found a suitable model in mouse mammary tumors induced by the mammary tumor virus (MTV). High frequencies of mice expressing the virus spontaneously develop mammary tumors after lactation, and a variety of murine mammary tumor lines have been established. Moreover, monoclonal antibodies against the major MTV envelope glycoprotein, gp52, have been raised, and have been found to bind specifically to tumor cell surfaces.

We have found using flow cytometry, that a mAb against viral gp52, P2AE12, binds to the surfaces of spontaneous mammary tumors and to cultured mammary tumor lines, but little or none to spleen cells. When crosslinked to an anti-murine CD3 mAb, the P2AE12 x anti-CD3 bispecific antibody induces murine T cells to lyse mammary tumor lines and spontaneous mammary tumors, and blocks the growth of mammary tumor cells in culture. Therefore, we are pursuing our evaluation of the mouse mammary tumor model as a totally syngeneic murine system for studying immune targeting with bispecific antibodies in vivo.

Proposed course of project

We envision that most of our work will focus on the mammary tumor system. We will develop in vivo tumor models that grow and metastasize in a reproducable

fashion. We are currently producing hybrid-hybridoma antibodies from P2AE12 and anti-CD3, and plan to make genetically engineered single chain bispecific antibodies from these same mAbs. The fates of these bispecific antibodies will be followed in normal and tumor bearing mice, and studies will be done to see whether these antibodies can block tumor growth in such mice. We will be especially interested to see whether bispecific antibodies can exert an anti-tumor effect in mice bearing spontaneous mammary tumors.

With regard to the xenogeneic ovarian carcinoma system, we currently have no specific plans for future experiments. However, investigators in the Medicine Branch of the NCI are currently developing protocols to test the Centocor anti-CD3 x anti-ovarian carcinoma hybrid-hybridoma in ovarian cancer patients. As that study progresses, we will test various aspects of the protocol in our mouse model for efficacy and for adverse effects.

Publications:

Garrido MA, Valdayo MJ, Winkler DF, Titus JA, Hecht TT, Perez P, Segal DM, Wunderlich JR. Targeting human T lymphocytes with bispecific antibodies to react against human ovarian carcinoma cells growing in nu/nu mice. Cancer Res 1990;50:4227-4232.

Segal DM. The use of flow cytometry to measure cell-cell interactions and conjugate formation. Current Protocols In Immunology. Vol 1 Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM and Strober W eds. John Wiley & Sons, New York, Chapter 5, pp 5.6.1-5.6.8. (1991)

Segal DM. Antibody mediated killing by leukocytes. In: Fc Receptors and the Action of Antibodies, H. Metzger (ed.). American Society for Microbiology, Washington, D.C.,1990 pp291-301.

Garrido MA, Valdayo MJ, Winkler DR, Titus JA, Hecht TT, Perez P, Segal DM, Wunderlich JR. Refocussing the immune system to react with human tumors by targeting human lymphocytes with bispecific antibodies. Develop biol Standard 1990;71:33-42.

Braakman E, Goedegebuure PS, Vreugdenhil RJ, Segal DM, Shaw S, Bolhuis RLH. CAM-melanoma cells are relatively resistant to CD3-mediated T-cell lysis targeting. Int.J.Cancer 1990;46:475-480

Donohue JH, Ramsey PS, Kerr LA, Segal DM, McKean DJ. Enhanced in vitro lysis of human ovarian carcinomas with activated peripheral blood lymphocytes and bifunctional immune heteroaggregates. Cancer Research 1990;50:6508-6514.

Segal DM, Garrido MA, Qian J-h, Mezzanzanica D, Andrew SM, Perez P, Kurucz I, Valdayo MJ, Titus JA, Winkler DF, Wunderlich JR. Effectors of targeted cellular cytotoxicity. Molec Immunol 1990;27:1339-1342.

Fanger MW, Segal DM, Romet-Lemonne J-L. Bispecific antibodies and targeted cellular cytotoxicity. Immunol Today 1991;12:51-54

Goedegebuure PS, Braakman E, Segal DM, Vreugdenhill RJ, Bolhuis RLH. Lymphocyte leukocyte function-associated antigen 1 interacting with target cell intercellular adhesion molecule 1 co-activates cytolysis triggered via CD16 or the receptor involved in major histocompatibility antigen-unrestricted lysis. International Immunol 1990;2:1213-1220.

Qian J-h, Titus JA, Andrew SM, Mezzanzanica D, Garrido MA, Wunderlich JR, Segal DM. Human PBL targeted with bispecific antibodies release cytokines that are essential for inhibiting tumor growth. J Immunol 1991;146:3250-3256.

Segal DM, Qian J-h, Andrew SM, Titus JA, Mezzanzanica D, Garrido MA, Wunderlich JR. Cytokine release by PBL targeted with bispific antibodies, and its role in blocking tumor growth. Annal NY Acad Sci in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09255-17 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Application of Flow Cytometry to Cell Biology

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

PI:	S. O. Sharrow	Senior Investigator	EIB, NCI
Others:	M. A. Sheard	Biologist	EIB, NCI
	L. G. Granger	Biologist	EIB, NCI

Members of the Experimental Immunology Branch, NCI (see text)

COOPERATING UNITS *(if any)*

A. Schultz, L. Barden, and R. Tate, CSL, DCRT; C. C. Ting, OD, DCBDC, NCI.

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The Experimental Immunology Branch flow cytometry laboratory currently supports multiple research projects for more than 55 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.

PHS 6040 (Rev 1/84)

U.S GOVERNMENT PRINTING OFFICE: 1991 O—864-939

Project Description

Major Findings:

The EIB flow cytometry laboratory operates and maintains a dual-laser flow cytometer and associated ADP equipment, maintains and provides training for three user-operated single beam flow cytometers, maintains a reagent bank which supplies reagents to users of the flow cytometers, and provides consultation in flow cytometry techniques, protocol design, reagent selection, and data analysis. This report summarizes findings only in selected project areas which utilized the dual-beam flow cytometer, and emphasizes those aspects most heavily supported by the use of flow cytometry analysis.

Dr. A. Singer and colleagues utilized flow cytometry analysis in a series of investigations involving in vivo and in vitro analyses of intra-cellular signalling via murine T cell accessory molecules. It was found that in vivo treatment with anti-CD4 monoclonal antibody resulted in increased cell surface expression of T cell receptor on CD4,CD8 double positive thymocytes. This effect was dependent upon the maturational state of developing T cells as anti-CD4 treatment resulted in decreased T cell receptor expression on the surface of mature T cells. These investigators have also found that cell surface T cell receptor expression increases on immature thymocytes when these cells are released from the thymic micro-environment, and that this increase can be blocked by anti-CD4 but not anti-CD8 antibody. Because Class II molecules are known to be a ligand for CD4, the effect of Class II positive stimulator cells upon T cell receptor induction was tested. It was found that; a) Class II expressing cells could indeed block T cell receptor induction, b) this blockade was reversed by anti-Class II monoclonal antibody, and c) induction was inhibited on all $CD4^+CD8^+$ double positive thymocytes regardless of the Class II haplotype of either the thymocytes or the Class II positive stimulator cells. Together these results suggest that during ontogeny the CD4 accessory molecule may function to regulate T cell receptor expression and function on developing T cells via interactions with monomorphic Class II determinants expressed on thymus epithelial and/or dendritic cells.

Dr. A. Singer and colleagues have also utilized flow cytometry in studies of intra-thymic T cell differentiation and the characterization of phenotypically distinct thymic subsets. In one of these studies a novel, low frequency, subpopulation expressing an unusual T cell receptor (TCR) repertoire was identified by high cell surface expression of the Ly-6C antigen. This subset was found to appear late in ontogeny and to express a skewed TCR repertoire characterized by overexpression of Vβ8 and expression of potentially autoreactive T cell receptors. Unlike $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes which share these characteristics, $Ly\text{-}6C^+$ thymocytes were found to contain all four subpopulations defined by CD4 and CD8 expression, including the $CD4^+CD8^-$ mature thymocyte subset. It was also found that during their development, $Ly\text{-}6C^+$ thymocytes progress through the CD4/CD8 developmental pathway in an ordered sequence identical to that of conventional $Ly\text{-}6C^-$ thymocytes. Taken together, these results suggest that $Ly\text{-}6C^+$ thymocytes are the precursors of $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes, and that $Ly\text{-}6C^+$ thymocytes may represent a distinct lineage within intra-thymic T cell differentiation.

Dr. A. Singer and colleagues utilized flow cytometry in studies which examined the expression of CD4 and CD8 accessory molecules on the surface of thymocytes conventially thought to be $CD4^-CD8^-$ (double negative). This investigation was prompted by the initial observation that, when analyzed with the exquisitely sensitive technique of flow cytometry, "double negative" thymocytes actually expressed low levels of CD4 and CD8 accessory molecules. To examine the possibility that these accessory molecules were acquired, rather than synthesized, artificial in vivo and in vitro mixes of thymocytes expressing distinguishable alleles of CD8 were examined. It was found that both CD4 and CD8 molecules could be passively acquired by thymocytes from their environment, and that in vitro acquisition of CD8 molecules was enhanced by the expression of Class I MHC. These studies demonstrate that caution must be exercised in the interpretation of the significance of low level expression of CD4 and CD8 on developing thymocytes.

Dr. Shearer and colleagues have used flow cytometry to study the repopulation of the immune system by donor lymphoid cells during graft-versus-host (GVH) reactions induced by the injection of parental lymphocytes into unirradiated adult F_1 mice. Analysis of host and donor populations during both the acute immunosuppresive phase of GVH, as well as during gradual recovery, demonstrated a complex pattern of changes in lymphoid and myeloid populations that eventually resulted in the repopulation of the host with donor derived lymphohematopoietic cells. Initially, donor-derived T cells, especially $CD8^+$ cells, expanded, followed by disappearance of both T and B cell host populations. Gradually, the lymphohematopoietic system was reconstituted with donor-derived cells in an orderly sequence of myeloid populations followed by B cells, and eventually T cells. The recovery of immune functions was associated with repopulation of the spleen with these donor-derived T cells. Full donor repopulation required the presence of both $CD4^+$ and $CD8^+$ T cells in the parental cell inoculum. Depletion of donor $CD4^+$ T cells abrogated development of GVH and all donor engraftment, while depletion of $CD8^+$ T cells resulted in engraftment of only donor $CD4^+$ T cell subpopulations. These results demonstrate that the recovery of immune function following graft-versus-host disease is due to repopulation of cells derived from the graft donor and have implications for our understanding of bone marrow transplantation therapy.

Dr. Hodes and colleagues employed flow cytometry in an exhaustive series of analyses of expression of the repertoire of T cell receptor genes in inbred strains, recombinant inbred strains, backcross animals, and tumor infiltrating lymphocytes (TIL). These studies have focussed on; a) characterization of associations between Mls stimulatory antigen expression and the expression of specific T cell receptor Vβ gene products, b) analysis of negative selection of the T cell repertoire, and c) analysis of ligands mediating Vβ-specific negative selection of the T cell repertoire It was found that there is an association between Mls expression and clonal deletion in both the thymus and the periphery of Mls-reactive T cells. This negative selection of the T cell repertoire was thymus dependent and did not occur in athymic nude mice. When the self ligands responsible for deletion were analyzed, it was found that both MHC and non-MHC antigens appear to be involved. Based upon these analyses, new superantigens have been proposed on the basis of clonal deletion of Vβ11, Vβ12, and Vβ5. In

other studies the Vβ TCR repertoire of tumor infiltrating lymphocytes (TIL) from syngeneic tumors was analyzed. Freshly isolated TIL were found to include both $CD4^+$ and $CD8^+$ positive T cells, as well as cells with NK markers. These cells were predominantly $TCR\alpha\beta^+$ And expressed multiple Vβ gene products. While restricted Vβ expression developed during in vitro culture, no dominant pattern emerged. The complexity of TCR usage in an anti-tumor response may result from the involvement of multiple α- and β-chain regions in the response to a single antigenic determinant, or may reflect multiple antigenic determinants expressed on a single syngeneic tumor. These studies have expanded our knowledge of the mechanisms whereby the T cell receptor repertoire is generated and extended our understanding of the nature of T cell recognition for Mls gene products.

Dr. Hodes and colleagues have also utilized flow cytometry in studies of B cell activation. A novel activated B cell subpopulation induced by IL-5 was identified by high expression of Pgp-1 (CD44). These $CD44^{hi}$ B cells exhibited low expression of Class II and B220 (CD45), and were found to bind hyaluronate in a Pgp-1 dependent manner. It was further demonstrated that $CD44^{hi}$ B cells were induced in mice undergoing chronic (stimulatory) graft-versus-host reactions. This novel activated B cell subset may play a role in immune tissue damage which occurs during GVH reactions.

Dr. Gress and colleagues utilized flow cytometry in a series of studies of bone marrow transplantation in mice, monkeys and humans. Flow cytometric analyses are used to characterize cell populations used in reconstitution, to monitor and characterize immune cell reconstitution, to analyze cellular components which contribute to rejection versus engraftment of stem cell populations used in reconstitution, and to evaluate immunosuppressive therapies used to prevent graft rejection. In a study of autologous transplantation in rhesus monkeys, it was found that the pattern and time course of reconstitution of T lymphocytes was dependent upon the number of residual T cells in the infused bone marrow, rather than upon the marrow dose or efficacy of general hematopoietic reconstitution. In a study of graft rejection in a murine model, it was demonstrated that cytotoxic T lymphocytes (CTL) were sufficient to reject allogeneic bone marrow grafts. Anti-CD3 antibody therapy and veto suppressor cell therapy which each inhibit CTL function were successfully used to prevent graft rejection. These studies are important to our understanding of the mechanisms of bone marrow engraftment and rejection, especially as applied to clinical problems.

Dr. Shaw and colleagues have extensively employed flow cytometry in extending their characterization of cell surface molecules which are differentially regulated on human T cells. Initial investigations had demonstrated that functionally distinct T cell subsets express quantitatively different surface levels of multiple biologically functional molecules. Initial studies focussed on the $CD4^+$ T cell subset and demonstrated that unexpected large numbers of cell surface antigens are coordinately up-regulated or down-regulated as T cells differentiate between "virgin" (not previously stimulated), and "memory" (previously activated) states of maturation. It has also been found that cell surface proteins thought to be important in T cell migration are expressed at higher levels on memory T cells than on naive T cells. Recent studies have extended these analyses to $CD8^+$ T cells and to T cells from spleen, tonsil, and

lymph node, as well as blood. It was found that fewer $CD8^+$ than $CD4^+$ T cells exhibit a memory cell phenotype, regardless of the source of these cells. These analyses in progress are systematically characterizing the expression of over 400 cell surface markers on human T cells, and have implications for the role of regulated cell surface expression of adhesion molecules in the preferential migration of memory T cells into tissues.

S. Sharrow, L. Barden (CSL, DCRT) and colleagues have: a) installed ADP hardware and network systems to provide network communication and data processing hardware capabilities for new state-of-the-art flow cytometry instrumentation; b) installed, modified, and tested a new BDIS FACSTAR PLUS Dual Laser Flow Cytometer; c) installed and tested 6 Beta-Test versions of new data acquisition software designed to EIB flow cytometry laboratory specifications by BDIS; d) installed and tested three Beta-Test versions of protocol entry software designed to EIB flow cytometry laboratory specifications; e) further investigated, using the DCRT-developed Cluster Analysis Program (CAP), the application of automated cluster analysis techniques to flow cytometry multi-parameter data; and f) installed, tested, and modified new histogram analysis software for VAX/VMS systems which is derived from the DCRT-developed Laboratory Analysis Package (LAP).

In one of these studies, cluster analysis identified subpopulations of murine thymocytes which were not recognized using conventional histogram techniques. These novel cells express high levels of T cell receptor (TCR), a phenotype characteristic of mature T cells. However, these cells also express both the CD4 and CD8 accessory molecule, a phenotype (double positive) which is characteristic of immature thymocytes. Interestingly, the pattern of CD4/CD8 expression of these TCR-high double positive thymocytes is distinct from that found on other immature thymocytes. TCR-high double positive thymocytes contain at least 2 subpopulations: a) one which expresses high levels of CD4 and low levels of CD8; and b) one which expresses high levels of CD8 and low levels of CD4. It was further demonstrated that the T cell receptor repertoire of this TCR^{hi} $CD4^+CD8^+$ subset is analagous to that of mature T cells, which have undergone repertoire selection, rather than to that of immature thymocytes. This novel subpopulation of thymocytes may represent cells which, as a consequence of differentiation events, is in transition between immature and mature thymocytes. Study of these cells may permit analysis of the mechanisms which control repertoire selection.

Development and modifications of flow cytometric hardware and software have been performed with the goal of improving the functionality, flexibility, user interface, and throughput of commercially available and non-proprietary hardware and software. During the past year, the flow cytometry laboratory has implemented direct network access to flow cytometry data and software for members of the EIB. Using either IBM-compatible or Macintosh pc's these users now have the capability to analyze either previously stored, or just collected, flow cytometry data. A program has been developed which allows users, via network connections, to construct storable, editable, protocols for flow cytometry experiments. Most importantly, data acquisition software has been developed which utilizes files created by the protocol generation software. This permits automated insertion into text blocks of previously-entered investigator-created sample information. A variety of hardware and software modifications have been

implemented to provide sample throughput capacity which is limited only be sample input, and not by interaction with computer software.

Publications:

Nakayama T, June CH, Munitz TI, Sheard M, McCarthy SA, Sharrow SO, Samelson LE, Singer A. Inhibition of T cell receptor expression and function in immature $CD4^+8^+$ thymocytes by CD4. Science 1990;249:1558-1561.

Ting C-C, Hargrove ME, Henrich JP. Anti-CD3 antibody-induced activated killer cells. J. Immunological Inv. 1990;19:347-353.

Sharrow SO. Overview of flow cytometry. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. eds.: Current Protocols in Immunology. New York Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, 1991;5.1.1-5.1.8.

Sharrow SO. Analysis of flow cytometry data. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W., eds.: Current Protocols in Immunology. New York Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, 1991;5.2.1-5.2.10.

Hakim FT, Sharrow SO, Payne S, Shearer GM. Repopulation of host lymphohematopoietic systems by donor cells during graft-versus host reaction in unirradiated adult F_1 mice injected with parental lymphocytes. J Immunol 1991;146:2108-2115.

Guy R, Foo-Phillips M, Sharrow SO, Hodes RJ. Subpopulations of fetal thymocytes defined by expression of T cell receptor/CD3 and IL-2 receptor: CD3 and IL-2 receptor alpha-chain are expressed on reciprocal cell populations. J Immunol 1991:146:418-424.

Shores EW, Sharrow SO, Singer A. Presence of CD4 and CD8 determinants on CD4-CD8- murine thymocytes: passive acquisition of CD8 accessory molecules. Eur J Immunol 1991;21:973-977.

Karpati RM, Banks SM, Malissen B, Rosenberg SA, Sheard MA, Weber JS, Hodes RJ Phenotypic characterization of murine tumor-infiltrating T lymphocytes. J Immunol 1991;146:2043-2051.

Vacchio MS, Ryan JJ, Hodes RJ. Characterization of the ligand(s) responsible for negative selection of $V\beta11$- and $V\beta12$-expressing T cells: Effects of a new Mls determinant. J Exp Med 1990;172:807-813.

Abe R, Foo-Phillips M, Hodes RJ. Genetic analysis of the Mls system: Formal Mls typing of the commonly used inbred strains. Immunogenetics 1991;33:62-73.

Murakami S, Miyake K, June CH, Kincade PW, Hodes RJ. IL-5 induces a Pgp-1 (CD44) bright B cell population that is highly enriched in proliferative and Ig secretory activity and binds to hyaluronate. J Immunol 1990;145:3618-3627.

Murakami S, Miyake K, Abe R, Kincade PW, Hodes RJ. Characterization of autoantibody-secreting B cells in mice undergoing stimulatory (chronic) GVH reactions: Identification of a $CD44^{hi}$ population that binds specifically to hyaluronate. J Immunol 1991;146:1422-1427.

Maguire JE, McCarthy SA, Singer A, Singer DS. Inverse correlation between steady-state RNA and cell surface T cell receptor levels. FASEB J 1990;4:3131-3134.

Nakamura H, Gress RE. Interleukin 2 enhancement of veto suppressor cell function in T-cell-depleted bone marrow in vitro and in vivo. Transplantation 1990;49:931-937.

Hirsch R, Bluestone JA, DeNenno L, Gress RE. Anti-CD3 $F(ab')_2$ fragments are immunosuppressive in vivo without evoking either the strong humoral response or morbidity associated with whole mAb. Transplantation 1990;49:1117-1123.

Hirsch R, Gress RE, Bluestone JA. Anti-CD3-mediated immunotherapy: A murine model. In: Burlinghan, W. ed. Critical Evaluation of Antibody Therapies in Transplantation. Boca Raton CRC Press:in press.

Snider DP, Uppenkamp IK, Titus JA, Segal DM. Processing fate of protein antigen attached to IgD or MHC molecules on normal B lymphocytes using heterocrosslinked bispecific antibodies. Molec Immunol in press.

Tanaka Y, Shaw S, Horgan KJ. Human naive and memory T cells. Med. Immunol 1991;in press.

Abe R, Kanagawa O, Sheard MA, Malissen B, Foo-Phillips M. Characterization of a new minor lymphocyte stimulatory system. I. Cluster of self antigens recognized by "I-E-reactive" Vβs, Vβ5, Vβ11, and Vβ12 T cell receptors for antigen. J Immunol 1991;in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09257-17 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Mechanisms of Cellular Immune Responses

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

PI:	Stephen Shaw	Section Chief	EIB, NCI
Others:	Gale Ginther-Luce	Chemist	EIB, NCI
	John Hallam	Biologist	EIB, NCI
	Gij van Seventer	Visiting Associate	EIB, NCI
	Yoshiya Tanaka	Visiting Fellow	EIB, NCI
	Tamas Schweighoffer	Visiting Fellow	EIB, NCI
	Marina Giunta	Visiting Fellow	EIB, NCI
	David Adams	Special Volunteer	EIB, NCI

COOPERATING UNITS *(if any)*

Otsuka Pharmaceuticals: Walter Newman, University Hospital, London Ontario: Andrew Lazarovits, Cleveland Clinic Foundation: Claudio Fiocchi

LAB/BRANCH

Experimental Immunology Branch

SECTION

Human Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.9	7.9	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Our studies continue to emphasize two fundamental areas: 1) identifying and characterizing the functions of cell surface molecules which facilitate T cell recognition; and 2) analysis of heterogeneity among subsets of human T cells and of the functional capacities of those subsets. Using multicolor flow cytometry, we have elucidated simplifying principles regarding changes in expression of two classes of molecules during CD4 T cell development which are critical to T cell adhesion: integrins which mediate adhesion and trigger molecules which regulate that adhesion. Among naive T cells there is low homogenous expression of integrins $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\beta 1$. However, among memory cells there is augmented expression of one or more of these integrins and marked heterogeneity. Among the multiple memory cell subsets distinguished by differential expression of $\alpha 4$ and $\beta 1$, we have emphasized studies of a subset with high $\alpha 4$ but low $\beta 1$ expression. Multiple lines of evidence indicate that these represent gut-homing cells, especially our finding that they have uniquely high expression of the integrin $\alpha 4\beta 7$. We have continued our studies of the CD31 molecule with its unique capacity to regulate T cell adhesion. CD31 shows variation in expression of T cells in various secondary lymphoid tissue and nonlymphoid tissue, consistent with a role in regulating T cell migration. Further, it has multiple effects on T cell activation indicating a role in activation of both T cell and monocytes. Finally, we have analyzed early signaling events involved in T-cell activation to determine the contribution by LFA-1/ICAM-1 interaction; these studies demonstrate that it leads to signal transducing events resulting in prolonged phospholipase C (PLC) activation and PIP_2 hydrolysis, and a sustained increase in $[Ca^{2+}]_i$ level.

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U S GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description

Major Findings

Integrin molecules are critical to many aspects of T cell adhesion and function; we have previously noted differences in their expression on naive and memory cells and demonstrated functional differences consistent with these phenotypic differences. In an extension of that past analysis, we undertook systematic flow cytometric analysis of surface expression of the CD45RA and CD45RO isoforms that distinguish naive and memory cells and of 5 VLA integrin chains on CD4 T cells. These studies reveal some simple relationships. There is virtually perfect reciprocity between CD45RA and CD45RO isoform expression not only by CD4 T cells in circulation, but also in spleen and lymph node. Furthermore, among circulating CD4 T cells, the expression of VLA-β1 (CD29) and of VLA-α3, -α5, and -α6 are strongly correlated. In contrast, other inter-relationships follow complex patterns. CD45RO expression and VLA-β1 expression correlate only moderately in circulating CD4 T cells and poorly among CD4 T cells from lymph node and spleen. VLA-α4 expression (unlike VLA-α3, VLA-α5, and VLA-α6) shows substantial discordance with VLA-β1 expression.

The critical analysis reduces to understanding the relationships between CD45RO, VLA-β1 and VLA-α4, which can be summarized as follows. $CD45RO^-$ cells, generally understood to be naive cells, are rather homogeneous with respect to low expression of both VLA-α4 and VLA-β1. In marked contrast, $CD45RO^+$ cells, generally understood to be memory cells, show marked heterogeneity, especially when the markers VLA-α4 and VLA-β1 are analyzed together. All the $CD45RO^+$ cells have increased levels (relative to $CD45RO^-$ cells) of VLA integrins: many show increased levels of both VLA-α4 and -β1; however, some have increased levels principally of either VLA-β1 or VLA-α4. These results establish that: 1) regulation of VLA-α4 can occur independently of VLA-α3/VLA-α5/VLA-α6/VLA-β1 and 2) VLA-α4 and VLA-β1 are important discriminators of subsets of $CD45RO^+$ memory cells.

Thus, there is striking diversity among memory CD4 T cells in their integrin phenotype. This provides a potential molecular explanation for the observations of highly selective patterns of homing of memory cells to particular tissues. Of particular interest is our finding of a subset of CD4+ memory T lymphocytes has an unexpected VLA-$\beta 1^{low}\alpha 4^{high}$ integrin phenotype. By an exhaustive screening of more than 400 mAb from multiple sources, we have identified a unique mAb, Act-1, which identifies this subset. Immunoprecipitation and ELISA assays demonstrate that Act-1 binds the a4b7 integrin molecule. $\alpha 4\beta 7$ is involved in binding to both fibronectin and VCAM-1; Act-1 blocks cell binding to the former and augments binding to the latter. Act-1 expression marks a subset of memory cells which, unlike the predominant circulating memory cell, has upregulated $\beta 7$ rather than $\beta 1$. Their phenotype is distinct from that described for skin-homing T cells and is fully consistent with gut homing. Differential adhesion capacity of this subset is verified by selective binding to FN and VCAM-1 in a $\beta 1$-independent fashion. Thus, $\alpha 4\beta 7$ detected

on this subset by mAb Act-1 fits the expectations for a functional gut-homing receptor.

We have also been investigating an important functional class of molecules on T cells which are able to modify the adhesive capacity of those T cells; we designate such molecules "triggers" because they are able to induce functional activation of the integrin molecules on those T cells. We have identified three additional molecules which have that capacity: CD7, CD28 and CD31. We have placed particular emphasis on studies of CD31, since three of its features distinguish it from other trigger molecules on T cells: sensitivity to bivalent crosslinking, selectivity of induction of VLA-4 adhesion (relative to LFA-1 adhesion) and definition of unique T cell subsets which express it. Studies of the in vitro functional activities of CD31 mAb demonstrate complex multifactorial effects on T cell function and activation, as well as apparent effects on macrophages. Furthermore, differences in expression and function of different epitopes defined by monoclonal antibodies suggest that CD31 is a complex multifunctional structure involved in regulating interactions of multiple cell types with their extracellular environment.

After laying extensive foundation with our studies of phenotypes of T cells in peripheral blood, we have begun exploring expression in lymphoid and non-lymphoid tissue. Unfortunately, our hopes that lymph node and spleen might provide simple answers have so far been disappointed; this fits with the great complexity of phenotypes in peripheral blood. Perhaps most surprising have been recently initiated studies of gut lamina propria lymphocytes provided by a collaboration with Dr. C. Fiocchi. Although the analysis is by no means simple, it is clear that the phenotype of gut lamina propria T cells differs from circulating T cells in many ways. Our results confirm those of others, that the LPL are mostly $CD45RO^{+}$ and therefore presumptively memory cells; in addition there is a tendency for expression of activation markers, although only some of the classic activation markers are elevated suggesting a selective rather than global activation stimulus. We are actively pursuing phenotypic and functional analyses of tissue T cells in the belief that careful analysis will allow us to unravel their complexities, as it has enabled us to do with peripheral blood.

We have initiated a similar systematic analysis of cultured tumor-infiltrating lymphocytes (TIL) in collaboration with Dr. J. Yannelli in the NCI Surgery Branch. We expect that careful analysis will facilitate understanding of the physiology of TIL and the design of adoptive immunotherapy to optimize migration and function of TIL. Our analysis already demonstrates dramatic differences between the surface phenotype of these cells and that of normal circulating T cells. We are systematically exploring the molecular mechanisms for TIL binding to HUVEC cells. Thus far the same general rules pertain as have been observed with resting CD4 cells. The most striking difference is the fact that the integrins on TIL are already in a functionally activated state and show little further

augmentation in binding when activated by various agents. Subtle hints of additional pathways of adhesion have proved somewhat elusive.

We continue to study the capacity of adhesive interactions to facilitate T cell activation. We analyzed early signaling events involved in T-cell activation to determine the contribution by LFA-1/ICAM-1 interaction. We studied in detail the hydrolysis of phosphatidylinositol-(4,5)-biphosphate (PIP_2) and intracellular levels of free Ca^{2+} ($[Ca^{2+}]_i$) during stimulation with beads coated with the CD3 mAb OKT3 alone or in combination with purified ICAM-1 protein. Our investigations show no response to LFA-1/ICAM-1 alone, but that costimulation by LFA-1/ICAM-1 interaction induces prolonged inositol phospholipid hydrolysis (up to 4 hr) resulting in generation of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ and their derivatives. Based on studies with cycloheximide this costimulatory effect of prolonged inositol phospholipid hydrolysis appears dependent in part on de novo protein synthesis. A sustained increase in $[Ca^{2+}]_i$ level is also observed after LFA-1/ICAM-1 costimulation, which is at least partly dependent on extracellular sources of Ca^{2+}. Kinetic studies indicate that costimulation requires a minimal period of 4 hours of LFA-1/ICAM-1 interaction to provide maximal costimulation for OKT3-mediated T-cell proliferation. Thus, the necessary costimulation required for OKT3-mediated proliferation in this model system may be provided by an extended LFA-1/ICAM-1 interaction that in combination with OKT3 mAb leads to signal transducing events resulting in prolonged phospholipase C (PLC) activation and PIP_2 hydrolysis, and a sustained increase in $[Ca^{2+}]_i$ level.

Publications:

Ockenhouse CF, Ho M, Tandon NN, van Seventer GA, Shaw S, White NJ, Jamieson GA, Chulay JD, Webster HK. Molecular basis of sequestration in severe and uncomplicated Plasmodium Falciparum malaria: Differential adhesion of infected erythrocytes to CD36 and ICAM-1. J Infect Dis 1991;164:163-169.

van Seventer GA, Shimizu Y, Horgan KJ, Luce GEG, Webb D, Shaw S. Remote T-cell costimulation via LFA-1/ICAM-1 and CD2/LFA-3: demonstration with immobilized ligand/mAb and implication in monocyte-mediated costimulation. Eur J Immunol 1991;21:1711-1718.

Shimizu Y, Shaw S. Lymphocyte adhesion mediated by VLA (β1) integrins: Functional roles of multiple extracellular matrix and cell surface ligands. Chem Immunol 1991;50:34-54.

van Seventer GA, Shimizu Y, Shaw S. Roles of multiple accessory molecules in T-cell activation. Curr Opin Immunol 1991;3:294-303.

Shimizu Y, Newman W, Gopal TV, Horgan KJ, Graber N, Beall LD, van Seventer GA, Shaw S. Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1 and ELAM-1 and changes in pathway hierarchy under different activation conditions. J Cell Biol 1991;113:1203-1212.

Tanaka Y, Shaw S, Horgan KJ. Human naive and memory T cells subsets. Med Immunol 1991;21:527-540.

van Seventer GA, Newman W, Shimizu Y, Nutman TB, Tanaka Y, Horgan KJ, Gopal TV, Ennis E, O'Sullivan D, Grey H, Shaw S. Analysis of T-cell stimulation by superantigen plus major histocompatibility complex class II molecules or by CD3 monoclonal antibody: costimulation by purified adhesion ligands VCAM-1, ICAM-1 but not ELAM-1. J Exp Med 1991;174:901-913.

Shimizu Y, Newman W, Tanaka Y, Shaw S. Lymphocyte interactions with endothelial cells. Immunol Today 1992;13:106-112.

Shimizu Y, van Seventer GA, Ennis E, Newman W, Horgan KJ, Shaw S. Crosslinking of the T cell-specific accessory molecules CD7 and CD28 modulates T cell adhesion. J Exp Med 1992;175:577-582.

Shimizu Y, Tanaka Y, Shaw S. T cell adhesion molecules: relevance to autoimmunity. In: Bona CA, Siminovitch K, Theofilopoulos AN, Zanetti M, eds. The molecular pathology of autoimmunity. New York: Harwood Academic Publishers. 1992, in press.

Tanaka Y, Albelda SM, Horgan KJ, van Seventer GA, Shimizu Y, Newman W, Hallam J, Newman PJ, Buck CA, Shaw S. CD31/PECAM-1 is a preferential amplifier of VLA-integrin-mediated adhesion for distinctive T cell subsets. J Exp Med. 1992, in press.

Horgan KJ, Tanaka Y, Shaw S. Post-thymic differentiation of CD4 T lymphocytes: Naive vs memory subsets and further specialization among memory cells. Prog Chem Immunol. 1992, in press.

Tanaka Y, Shaw S. T cell adhesion cascades: General considerations and illustration with CD31. In: Gupta S, Waldmann TA, eds. Mechanisms of Lymphocyte Activation and Immune Regulation IV: Cellular Communication. New York: Plenum Publishing Corp. 1992, in press.

Horgan KJ, Shaw S. Immunological memory. In: Roitt IM, ed. Encyclopedia of Immunology. London: Sanders Scientific Publications. 1992, in press.

Schweighoffer T, Shaw S. Concepts in adhesion regulation. In: Wegner CD, ed. Handbook of Immunopharmacology: Adhesion molecules. London: Academic Press. 1992, in press.

van Seventer GA, Shaw S. "Sticky" molecules are "tricky" molecules: The multiple roles of adhesion molecules in human T cell adhesion and activation. Int J Cancer 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09259-14 EIB

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October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Effects of Graft-versus-Host Reactions on Cell-Mediated Immunity

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

PI:	G.M. Shearer	Section Chief	EIB, NCI
Others:	F. Hakim	Senior Staff Fellow	EIB, NCI
	S. Sharrow	Senior Investigator	EIB, NCI
	A. Sher	Senior Investigator	LPD, NIAID
	R. Gazinneli	Visiting Fellow	LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Experimental Immunology Branch

SECTION
Cell Mediated Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

B

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

The graft-versus-host reaction (GVHR) induced by inoculating parental T cells into unirradiated F_1 hosts is a complex process. Donor repopulation of host lymphohematopoietic tissues requires both $CD4^+$ and $CD8^+$ donor T cells, and the immune dysregulation resulting from such a GVHR appears to involve a switch from a predominance of Th1 to Th2 type of helper function. By inducing a GVHR in a parent-F_1 combination in which the Mls locus would be recognized, we demonstrated that recognition of Mls determinants contributes to immune suppression in the parent-into-F_1 GVHR model, and contributes to mortality in irradiated mice given allogeneic bone marrow differing at Mls.

Studies of susceptibility and resistance of mice to infection with Toxoplasma gondii indicated that T. gondii-specific, $CD8^+$ mediated cytolysis is a chronic and acute GVHR, we found that both $CD4^+$ and $CD8^+$ T cell function is important in resistance to this parasite.

Studies of the affects of cyclosporin A on murine lymphopoiesis indicates that this immunosuppressive drug: a) selectively abrogates single-positive T cells; b) inhibits the development of TCR-expressing single-positive T cells; and c) augments natural killer cells.

U.S. GOVERNMENT PRINTING OFFICE: 1991 O-864 910

Project Description

Major Findings:

The graft vs host reaction (GVHR) generated by the injection of parental lymphocytes into unirradiated immune competent F1 hosts is characterized by an acute loss of immune functions, an attack on host tissues and a gradual recovery of function. Flow cytometric analysis of the donor-and-host-derived splenic populations during the course of acute dysfunction and gradual recovery revealed a complex pattern of changes in lymphoid and myeloid populations that resulted in the repopulation of the host with donor-derived cells. Initially donor-derived T cells populations expanded, particularly $CD8^+$ T cells, followed by the disappearance of host T and B populations. Finally, donor-derived cells repopulated the lymphohematopoietic system in the sequence of myeloid populations, B cells and, after a protracted period, T cells. The recovery of immune functions following GVHR-induced immune deficiency was associated with the repopulation of the spleen by donor-derived cells. Donor repopulation of the host lymphohematopoietic system required the presence of both $CD4^+$ and $CD8^+$ cells in the original donor inoculum. Depletion of donor $CD4^+$ populations precluded development of GVHR or any donor engraftment; depletion of $CD8^+$ cells resulted in engraftment solely of donor $CD4^+$ populations.

Induction of the acute, suppressive GVHR by injection or parental donor spleen cells into unirradiated F1 host mice results not only in an immediate deficit in all immune functions, but also in a long-lasting selective deficit in CD4 function. At 12 to 18 months after induction of GVHR, generation or cytotoxic T lymphocytes to hapten-modified syngeneic stimulators or to APC-depleted allogeneic stimulators was deficient, but could be recovered by addition of IL-2 production in response to hapten-modified syngeneic, class II MHC-disparate allogeneic or mitogen stimulation remained deficient for a long as 20 months after induction of GVHR. This Il-2 production defect was not due to low $CD4^+$ numbers, suppression or antigen presenting cell deficits. In contrast to the deficit in IL-2 production, CD4-dependent Il-4 production was enhanced in long-term GVHR. Thus the GVHR induced alteration in long term GVHR may involve a relative shift in CD4 cytokine production capacity from TH1 to TH2 cytokines, hence altering the responses to all stimuli.

Subsequent to acute suppressive GVHR in mice, the recovery of T cell dependent immune functions is delayed for several months and the development of a chronic GVHR is increased. We investigated whether new T cells matured after acute GVHR, and whether these were tolerant to host antigens. By 8-17 months after GVHR, the frequencies of splenic $CD4^+$ and $CD8^+$ T cells were found to be comparable to age-matched untreated host, although the lymphoid organ size and hence the total number of T cells was significantly reduced. Cells were unresponsive to host antigens in CTL assays, but did not suppress anti-host CTL responses. Finally, host-reactive $v_{\beta 11}$ TCR-expressing cells were found to

be clonally deleted from splenic $CD4^+$ and $CD8^+$ populations, suggestive of thymic negative selection. The evidence suggests that the post-GVHR thymus has the capacity to mature and educate $CD4^+$ and $CD8^+$ T cells and that failure to clonally delete self reactive populations is not a contributing factor to the development of chronic GVHR in this system. Injection of A/J splenocytes ($H\text{-}2D^d$, Mls^c) into unirradiated BAF_1 host mice ($H\text{-}2D^{d/k}$, Mls^d) results in an acute suppressive graft-vs-host-reaction (GVHR), characterized by immune dysfunction and appreciable donor cell engraftment; injection of the CBA/J parent ($H\text{-}2D^k$, Mls^d), which recognizes no Mls disparity in the host, results in little or no GVHR. Furthermore, the Mls^a-reactive $V_{\beta 6}$ and $V_{\beta 8.1}^+$ T cells subsets in A/J T cells expand significantly in the GVHR host. Finallym depletion of $V_{\beta 6}\pm$ and $V_\beta 8.1+$ T cells from the A/J population abrogates the proliferative response to BAF_1 in vitro and the development of GVHR in vivo. Thus, the response to Mls determinants can contribute to the generation of a GVHR (S. Muluk et al, manuscript submitted for publication).

Transplantation of allogeneic bone marrow and lymph node cells matched to the host at major histocompatibility loci, can generate a severe graft-vs-host disease (GVHD), charcterized by immune deficiency and early death. In transplants disparate for minor lymphocyte stimulating antigens (Mls), selective expansion of elements of the splenic T cell receptor V_β repertoire indicated that Mls reactivity might play an important role in early GVHD. One week after transplantation of BALB/c ($H\text{-}2^d$, Mls^c) cells into irradiated DBA/2 ($H\text{-}2^d$, Mls^a) hosts, 65% of the CD4 and 29% of the CD8 splenic T cells expressed the Mls^a-reactive $V_{\beta 6}$ and $V_{\beta 8.1}$ T cell receptors (compared with 16% and 7% respectively in syngeneic BALB/c transplants). To assess the dependence of GVHD upon Mls-reactivity, $V_{\beta 6}$ and $V_{\beta 8.1,2}$ expressing T cells were removed from the BALB/c donor inocula by antibody and magnetic bead treatment prior to injection into DBA/2 hosts. More than 90% of BALB/c-into-DBA/2 mice died by five weeks after undepleted transplants, but >70% survived at 15 weeks after V_β depleted transplants. Depletion of $V_{\beta 2}$ and $V_{\beta 14}$-expressing T cells from the donor BALB/c-C57BL/6 grafts (H-2 disparity) produced a marked expansion of donor T cells, but no selective expansion of $V_{\beta 6}$ or $V\beta_{8.1}$. Furthermore, C57BL/6 hosts died at 6-10 days, whether or not the donor BALB/c $V_{\beta 6}$ and $V\beta_{8.1,2}$ subsets had been depleted. Thus the depletion of donor T cell $V\beta$ subpopulations specific for host antigens can reduce the severity of GVHD (F. Hakim et al, manuscript submitted for publication).

Mice vaccinated with a live temperature sensitive mutant (TS-4) of Toxoplasma gondii develop complete resistance to subsequent challenge with a highly virulent toxoplasma strain (RH). Because $CD8^+$ T cells have been demonstrated to be critical to this protective immunity in vivo, the involvement of cytotoxic T lymphocytes in the killing of infected cells in vaccinated mice was investigated. Following restimulation in vitro, splenic T cells from vaccinated mice of either the BALB/c or C57BL/6 strains were found to kill syngeneic bone-marrow derived macrophages infected with TS-4 tachyzoites or pre-incubated with soluble T.gondii antigens. Unimmunized control mice or mice vaccinated with heat-killed TS-4 tachyzoites failed to generate

significant CTL activity in vitro. Moreover, the observed lytic reaction was found to be target-specific, not killing uninfected or unpulsed macrophages, even when included as bystanders in the assay. Target lysis did not depend on the production by the effector cells of either a cytotoxic supernatant factor or IFN-γ. Depletion of $CD8^+$ cells from the splenic effector cell population, however, abrogated the cytotoxic activity, whereas depletion of $CD4^+$ cells had little effect. The MHC restriction of the toxoplasma-specific cytolytic reaction was confirmed in studies employing effector cells from BALB/c mice and targets from congenic or mutant haplotype strains. These experiments indicated that target killing is primarily restricted by genes mapping within the H-2D/L^d loci. Together, these results establish MHC-restricted cytolysis as a major parameter of $CD8^+$ effector function against T.gondii and indicate that, in the case of this protozoan, antigen presentation to $CD8^+$ lymphocytes can occur as a result of either processing within infected cells or exogenous uptake of parasite antigens.

Toxoplasma-resistant strains of mice can normally survive a chronic with the ME-45 variant of T.gondii for more than one year, during which time the parasite is located primary in cysts in the brain. Resistance to T.gondii has been demonstrated to involve two mechanisms: first the production of IFN-γ, which activated macrophages to produce NO killing internal parasites, and second, the development of $CD8^+$ cytotoxic effectors which kill parasite-infected cells. We investigated the effect of the differing immune deficits of acute and chronic GVHR upon host resistance to chronic toxoplasmosis. Injection of B6 donor spleen cells into a B6D2F1 host produces an acute suppressive GVHR, characterized by expansion of host-reactive $CD4^+$ and $CD8^+$ lymphocytes, attack on host lymphohematopoietic tissues, and a profound suppression of all immune functions lasting several weeks. An acute suppressive GVHR in T.gondii infected B6D2F1 mice abrogated both generation of CTL against T.gondii-infected cells and the production of IFN-γ in response to T.gondii antigens. Brain cysts increased 60% by two weeks and 300% by four weeks, indicative of reactivation and expansion of the parasite. Significant mortality was observed between three and six weeks. A chronic GVHR, such as that induced by injection of DBA/2 spleen cells, is characterized by engraftment only of donor $CD4^+$ T cells and by selective suppression of host CD4 function. Chronic GVHR in T.gondii-infected B6D2F1 mice resulted in a marked decrease in the production of IFN-γ in response to T.gondii antigens or to mitogens. Unlike acute GVHR, however, these mice retained the capacity to generate CTL against T.gondii infected targets.

CyA is potent immunosuppressive agent that is used extensively used for the prevention of tissue allograft rejection. Some studies have reported, however, that CyA-treated animals can develop autoimmune graft-versus-host reaction. We have studied the effects of CyA on lymphopoiesis in mice, and demonstrated that: a) CyA selectively abrogated single positive $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ T cells; b) inhibition of the appearnce of TCR-$a\beta$-expressing single positive thymocytes (by fetal thymic organ culture); and augments natural ler

killer cells in bone marrow-transplanted mice treated with CyA (108).

Due to the similarities between the immune defects of parent-into-F1 GVHR and AIDS progression that we have developed during the past decade, we consider this project to be 70% AIDS-related.

Publications:

Gazzinell RT, Hakim FT, Hieny S, Shearer GM, and Sher, A. Synergistic role of $CD4^+$ and $CD8^+$ T lymphocytes in IFN- production and protective immunity induced by an attennuated Toxoplasma gondi vaccine. J Immunol 1991;146:286-292.

Kosugi A, and Shearer GM. Effect of cyclosporin A on lymphopoiesis. III. Augmentation of natural killer cell activity of bone marrow transplanted mice treated with cyclosporin A. J Immunol 1991;146:1416-1421.

Hakim FT, Payne S, and Shearer GM. Recovery of T cell populations after acute graft-vs-host reaction. J Immunol 1991, in press.

Hakim FT, Payne S, and Shearer GM. Selective reduction in $CD4^+$ TH1 activity following acute graft-vs-host reaction. J Immunol 1991, in press.

Muluk SM, Hakim FT, and Shearer GM. Regulation of graft-vs-host reaction by Mls^a reactive donor T cells. J Immunol, in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09263-09 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Programmed cell death in lymphocytes

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

PI:	P.A. Henkart	Senior Investigator	EIB, NCI
Others:	D. Cohen	Biotech Fellow	EIB, NCI
	R. Blumenthal	Microbiologist	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Experimental Immunology Branch

SECTION
Lymphocyte Cytotoxicity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	2.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In order to test the hypothesis programmed cell death in lymphocytes involves activation of an endogenous protease, we have tested the ability of several cysteine protease inhibitors to block the TcR-induced death of the $CD4^+$ T cell hybridoma 2B4. Two chemically distinct families of inhibitors of calpains and cathepsins, the E-64 and leupeptin families, were found to rescue 2B4 cells from death measured by trypan blue or propidium iodide uptake after overnight culture on immobilized α-CD3 or α-TcR antibodies. DNA breakdown measured by flow microfluorimetry was also blocked. These drugs enhanced the TcR-induced IL-2 secretion by as much as 9-fold, showing that they do not act by interfering with signal transduction. Other programmed cell death systems in lymphocytes tested were the steroid-induced death of $CD4^+CD8^+$ thymocytes, which was blocked by leupeptin family inhibitors, and the CH31 B cell line, whose mIgM-induced death was unaffected by these inhibitors. We have also found that short-term-cultured lymphocytes can undergo programmed cell death after TcR cross-linking. After activation of purified resting mouse lymph node T cells (or PNA^+ thymocytes) by immobilized α-CD3 or α-TcR antibodies, continuously dividing cells were be maintained in IL2 for periods of days to weeks. When re-exposed to surface-bound antibodies against TcR or CD3, these cells were found to undergo a decrease in cell division and mitochondrial metabolism, accompanyied by substantial cell death. When purified populations of $CD4^+$ and $CD8^+$ cells were cultured this way, the cell death effect was principally seen in the $CD4^+$ population, with more modest effects in all responses in the $CD8^+$ population. In the $CD4^+$ cells DNA breakdown was clearly seen to accompany cell death when analyzed by flow microfluorimetry. This effect was blocked by cyclosporin, co-culture with splenic macrophages, or cysteine protease inhibitors.

Project Description

Major Findings:

Given the findings in project #9251 that apoptotic cell death was induced by injection of proteases into cell cytoplasm, we formulated the hypothesis that programmed cell death involves activation of an endogenous protease. The endogenous proteases which seemed plausible are cysteine proteases: the lysosomal cathepsins and the calcium dependent calpains. We have thus tested the ability of several cysteine protease inhibitors to block the TcR-induced death of the $CD4^+$ T cell hybridoma 2B4. Two chemically distinct families of inhibitors of calpains and cathepsins, the E-64 and leupeptin families, were found to rescue 2B4 cells from death measured by trypan blue or propidium iodide uptake after overnight culture on immobilized α-CD3 or α-TcR antibodies. DNA breakdown measured by flow microfluorimetry was also blocked. These drugs enhanced the TcR-induced IL-2 secretion by as much as 9-fold, showing that they do not act by interfering with signal transduction. Other programmed cell death systems in lymphocytes tested were the steroid-induced death of $CD4^+CD8^+$ thymocytes, which was blocked by leupeptin family inhibitors, and the CH31 B cell line, whose mIgM-induced death was unaffected by these inhibitors. We have also found that short-term-cultured lymphocytes can undergo programmed cell death after TcR cross-linking. After activation of purified resting mouse lymph node T cells (or PNA^+ thymocytes) by immobilized α-CD3 or α-TcR antibodies, continuously dividing cells were be maintained in IL2 for periods of days to weeks. When re-exposed to surface-bound antibodies against TcR or CD3, these cells were found to undergo a decrease in cell division and mitochondrial metabolism, accompanyied by substantial cell death. When purified populations of $CD4^+$ and $CD8^+$ cells were cultured this way, the cell death effect was principally seen in the $CD4^+$ population, with more modest effects in all responses in the $CD8^+$ population. In the $CD4^+$ cells DNA breakdown was seen clearly when analyzed by flow microfluorimetry. This effect was blocked by cyclosporin, co-culture with splenic macrophages, or cysteine protease inhibitors. The in vitro steroid-induced programmed cell death of $CD4^+CD8^+$ thymocytes was found to be partially inhibited by leupeptin family protease inhibitors.

Proposed course:

The known intracellular cysteine proteases which are candidates for involvement in programmed cell death blocked by E64 and leupeptin are the lysosomal cathepsins and the calpains. The inhibitors act on both types of enzymes. We will introduce a specific calpain inhibitor, calpastatin, into 2B4 cells by osmotic lysis of pinosomes and see if it blocks the death response. We are also attempting to measurement calpain activation after TcR cross-linking. Other systems of programmed cell death are being looked at to see if they are susceptible to blocking by these protease inhibitors.

Publications:

Winslow SG, Henkart PA. Polyinosinic acid as a carrier in the microscale purification of total RNA. Nucl Acids Res 1991;19:3251.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09264-05 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Studies of T Lymphocyte Function in Transplantation

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

PI:	Gene M. Shearer	Section Chief	EIB, NCI
Others:	Mario Clerici	Visiting Associate	EIB, NCI
	Richard Schulick	Pratt Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

M. Weil, Dept. of Surgery, Univ. of Maryland School of Med., P. Kimmel, Renal Unit, George Washington Univ. School of Med., T.E. Starzl, Dept. of Trnsplnt Surgery, Univ. of Pitts., J. Burdick, The Johns Hopkins University School of Medicine

LAB/BRANCH

Experimental Immunology Branch

SECTION

Cell Mediated Immunity Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.3	1.5	0.8

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The in vitro T helper cell (Th) response of human peripheral blood leukocytes (PBL) to HLA alloantigens is mediated by three distinct Th-antigen presenting cell (APC) pathways: 1) $CD4^+$ Th and autologous APC (CD4-sAPC); 2) $CD4^+$ Th and allogeneic APC (CD4-aAPC); and 3) $CD8^+$ Th and allogeneic APC (CD8-aAPC). There is a hierarchy of sensitivity of these pathways to the immunosuppressive effects of cyclosporin A (CsA), such that the CD4-sAPC is the most sensitive and CD4-aAPC is the least sensitive, both in vitro and in vivo.

We tested the in vitro Th function of more than 250 human renal allograft recipients on multi-drug immunosuppressive therapy. Our results indicate that only the presence of an intact CD4-sAPC pathway correlated with chronic or acute graft rejection. Our findings suggest that this approach can be used to monitor the graft status of organ transplant recipients. This pattern was modified in patients who received simultaneous and pancreas grafts in that the incidence of rejection of both organs was increased to 100%, and all three Th pathways appeared to contribute to rejection.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description

Major Findings:

Past experience from several laboratories has indicated that in vitro tests of T cell function have not correlated well with solid organ allograft rejection in either experimental animals or humans. Two recent reports from this laboratory raised the possibility that only certain components of T cell immunity may be relevant for organ graft rejection. Thus, the Th response of human PBL to HLA alloantigens (ALLO) is complex and is mediated by three distinct Th-APC pathways: 1) $CD4^+$ Th that recognize ALLO processed and presented on autologous APC (sAPC); 2) $CD4^+$ Th; and 3) $CD8^+$ Th, both of which recognize ALLO on allogeneic APC (aAPC). We also observed that the CD4-sAPC pathway is the most sensitive, whereas the CD4-aAPC pathway is the least sensitive to the immunosuppressive effects of CsA, when the drug was added to ALLO-stimulated cultures of PBL from healthy donors. Based on the above findings, we proposed and tested the hypothesis that the CD4-sAPC pathway (but not the other pathways) would be important in the rejection of human solid organ allografts. By testing the PBL of more than 100 renal allograft recipients on different regimens of immunosuppressive drugs, we demonstrated that the CD4-sAPC pathway, but not the CD4-aAPC nor the CD8-aAPC pathways, is an important predictor of kidney graft rejection. Thus, all patients who were demonstrated to be undergoing chronic or acute rejection were shown to have a functionally intact CD4-sAPC pathway. Rejection was not associated with the presence of the other pathways, although when these were also suppressed, the patients were at risk for opportunistic infection. We have performed a long-term follow-up (3-4 years) of the five patients who exhibited no evidence of renal rejection, but nevertheless retained an intact CD4-sAPC pathway. Four of the five had lost all kidney function and were on dialysis, which could have been due to sub-clinical levels of chronic rejection that our test detected, but which were not noted by increased cretainine levels. We have also followed kidney graft recipients for six-month periods, and observed dynamic changes in the patterns of Th-APC responses. Nevertheless, those patients whose CD4-sAPC pathway was intact in more than 50% of the serial tests, were at high risk for subsequent renal failure, possibly due to sub-clinical episodes of chronic rejection.

Patients who received simultaneous kidney-pancreas transplants were at very high risk for rejection and their rejection events were associated with the Th pathways that involved allogeneic APC, in contrast to the renal allografts alone in which only the CD4-sAPC pathway appeared to relevant for rejection.

In collaboration with Dr. Thomas Starze (Univ. of Pittsburgh) we have studied 10 long-term surviving renal allograft recipients (26-29 years after transplant). All 10 of the patients appeared to be specifically donor-unresponsive to HLA alloantigens, despite the fact that some of them were receiving no immunosuppressive drugs.

Studies of patients who have received liver allografts suggest that all Th-APC pathways initially contribute to rejection. The study is continuing in longer-term recipients of liver grafts.

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:

Lucas PD, Shearer GM, Neudorf S, Gress RE. The human autoimmune xenogeneic cytotoxic response. I. Dependence on responder antigen-presenting cells. J Immunol 1990;144:4548-4554.

Muluk SC, Clerici M, Via CS, Weir MR, Kimmel PL, Shearer GM. A new approach for analysis of the mixed lymphocyte reaction that is predictive for human allograft rejection. Transpl Proc 1991;23:1274-1276.

Kosugi A, Shearer GM. Effect of cyclosporin A on lymphopoiesis. III. Augmentation of natural killer cell activity of bone marrow transplanted mice treated with cyclosporin A. J Immunol 1991;146:1416-1421.

Muluk SC, Clerici M, Via CS, Wier MR, Shearer GM. Selective loss of MHC self-restricted, $CD4^+$ T helper cell function in immunosuppressed kidney transplant recipients. Transplantation 1991;52:284-291.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09265-10 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Analysis of the T Cell Repertoire

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

PI:	R. J. Hodes	Section Chief	EIB, NCI
Others:	R. Abe	Visiting Associate	EIB, NCI
	M. Vacchio	Bio. Lab. Tech.	EIB, NCI
	S. Sharrow	Senior Investigator	EIB, NCI

COOPERATING UNITS *(if any)*

MB, NCI
LTI, NCI
University of Pittsburgh

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immune Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

When T cell receptor (TCR) expression was analyzed by flow cytometry and by mRNA quantitation, strain-specific decreases were detected in expression of 12 of the 22 known mouse Vβ products, representing negative selection of potentially self-reactive T cells in mice expressing the corresponding self ligands. These deleting ligands also functioned as "superantigens" capable of stimulating T cells expressing the corresponding Vβ products. Genetic mapping in all cases identified endogenous mouse mammary tumor (MMTV) proviruses encoding the Vβ-specific deleting ligands. These Vβ deletions fail to occur in athymic nude mice, demonstrating that the thymus is critical in tolerance by negative selection.

Exogenous viruses were analyzed for their influences on T cell repertoire. Milk-borne MMTV induced Vβ14 deletion only in strains of mice bearing natural or transgenic I-E class II major histocompatibility complex (MHC) product. A 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.

Although Vβ-specific superantigen effects are a model for the study of TCR selection, selection may more commonly depend on 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.

When TCR expression was analyzed in xenogeneic bone marrow transplantation between mouse and rat, it was found that tolerance to xenograft was accompanied by Vβ-specific T cell deletions. In the class I-restricted response of mouse $CD8^+$ T cells to HIV peptides, unqiue cross-reactive specificity patterns were accompanied by highly preferential use of specific Vβ products.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description

Major Findings:

1) Characterization of the Mls system of endogenous superantigens.

It was demonstrated that Mls^a and Mls^c are noncrossreactive and that the genes encoding Mls^a and Mls^c determinants are non-allelic and unlinked. An additional Mls determinant, Mls^f, was demonstrated, and is distinct from both Mls^a and Mls^c. Mls^a is the product of a single non-MHC gene. In contrast, Mls^c and Mls^f are both characterized by a novel "genetic redundancy", such that any one of two or more unlinked non-MHC genes determines an indistinguishable T cell determinant.

2) Negative selection in generation of the T cell receptor repertoire.

Generation of the T cell receptor repertoire involves negative selection as a means of deleting those T cells which are potentially reactive to self determinants. It was found that significant strain-specific decreases in expression occur in at least 12 of the 22 Vβ products and that each of these deletions is dominant in F_1 mice, consistent with the conclusion that these deletions occur in the process of eliminating T cells with potential reactivity for self determinants. The role of the thymus in mediating TCR negative selection was analyzed by studying congenitally athymic nude mice. A comparison of T cell receptor Vβ expression in congenic pairs of normal and athymic mice indicated that the normal Vβ deletions associated with tolerance to self products did not occur in athymic mice. These results demonstrate that the thymus has a critical role in mediating self tolerance by negative selection.

3) 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.

4) 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.

5) TCR expression in antigen-specific lymphocytes.

TCR expression was analyzed in freshly isolated TIL from mice bearing one of several antigenically distinct syngeneic tumors, or in in vitro lines derived from these TIL. TCR αβ expression was observed in the vast majority of TIL. Vβ usage was heterogeneous in these populations. The pattern of Vβ usage in TIL differed significantly from that observed in splenic T cells from the same strainbut no association was found between Vβ usage and the specificity of TIL for a particular syngeneic tumor. TCR Vβ expression was also analyzed in a panel of HIV peptide-specific $CD8^+$ cytotoxic T cell lines. In a panel of lines which recognize cross-reactive determinants on HIV peptides, a strong predominance of Vβ8 and Vβ14 expression was observed.

6) In vivo effects of exogenous retroviruses.

Milk-borne transmission of MMTV results in selective depletion of Vβ14-expressing T cells in the presence of appropriate MHC class II antigen. Another retrovirus, 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.

Proposed Course of Research:

1) Analysis of ligands mediating Vβ-specific negative selection.

a) Identification of MTV product. 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. Janet Butel (Baylor, Houston, TX), antibodies specific for MTV products, including the LTR product, will be 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.

Also in collaboration with Dr. Butel, products of MTV have been generated in a Baculovirus expression system. This material will be studied for its ability to mediate Vβ-specific T cell activation in vitro and clonal deletion or inactivation in vivo.

b) Mapping of additional Vβ ligands. The existence of Vβ-specific deleting ligands in species other than mouse, including human, will be studied by the technique of mixed bone marrow chimeras.

2) Selective TCR Vα/Vβ pairing.

It will be determined whether the observed non-random distribution of Vα/Vβ pairing reflects positive or negative immune selection of the T cell repertoire. Patterns of Vα/Vβ pairing will be determined in multiple inbred mouse strains. Where strain-specific patterns are observed, the genetic regulation of these patterns will be analyzed. The self ligands mediating any such selection will be identified initially by studies of genetic segregation.

3) 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.

b) In the MAIDS model of retroviral-induced immune deficiency, the nature of the observed T cell signaling abnormality will be analyzed further, together with structural characterization of the TCR complex and associated molecules.

Publications:

Guy R, Foo-Philips M, Sharrow SO, Hodes RJ. Subpopulations of fetal thymocytes defined by expression of T cell receptor/CD3 and IL-2 receptor: CD3 and IL-2 receptor α chain are expressed on reciprocal cell populations. J Immunol 1991;146:418-424.

Abe R, Kanagawa O, Sheard MA, Malissen B, Foo-Phillips M. Characterization of a new minor lymphocyte stimulatory system. I. Cluster of self antigens recognized by "I-E-Reactive" Vβs, Vβ5, Vβ11, and Vβ12 T cell receptors for antigen. J Immunol 1991;147:739-749.

Ildstad ST, Vacchio MS, Markus PM, Hronakes ML, Wren SM, Hodes RJ. Cross species transplantation tolerance: Rat bone marrow derived cells can contribute to the ligand for negative selection of mouse TCR-Vβ in chimeras tolerant to xenogeneic antigens (mouse + rat -->mouse). J Exp Med 1991; 175:147-157.

Vacchio MS, Kanagawa O, Tomonari K, Hodes RJ. Influence of T cell receptor Vα expression on Mls^a superantigen-specific T cell responses. J Exp Med 1992; 175:1405-1408.

Hodes RJ, Abe R. T cell recognition of Mls-like superantigens: Analysis of TCR requirements, superantigenic ligands, and signal transduction. Seminars in Immunology 1992:in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09266-10 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

T Cell Regulation of B Cell Activation

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

PI:	R. J. Hodes	Section Chief	EIB, NCI
Others:	K. Hathcock	Chemist	EIB, NCI
	H. Hirano	Visiting Fellow	EIB, NCI
	Q. Vos	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

Naval Medical Research Institute
Food and Drug Administration

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immune Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

C

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

IL5 induces B cell proliferation and immunoglobulin (Ig) secretion and results in appearance of a phenotypically novel B cell population which expresses high density of CD44 and low densities of B220 (CD45) and Ia.This B cell subpopulation mediates nearly all of the proliferative and Ig secretory activity of IL5-activated B cells. In addition, the CD44 expressed by these cells mediates binding to the extracellular matrix material hyaluronic acid (HA), indicating a potential role for CD44 in regulating trafficking of activated B cells in vivo. The CD44 expressed on IL5-stimulated B cells migrates with a lower molecular weight than does CD44 expressed by control B cells, reflecting differential glycosylation. 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. A series of mAb was generated by immunizing rats with activated mouse B cells. One of these mAb (GL7) reacts with a subpopulation of activated B cells, as well as with activated T cells. GL7 precipitates what appears to be a previously undescribed 29-31 KDa molecule from activated B cells. Another mAb generated in this fashion (GL1) reacts with activated B but not T cells. GL1 inhibits responses of $CD4^+$ T cells to activated B cells, suggesting that the target of GL1 may represent a costimulatory molecule for T cell activation.

To establish a system for the study of Th cell-B cell interaction at a single cell level, responses were generated using Ig transgenic B cells and cloned Th cells. Highly efficient hapten-specific responses were generated. The study of sera from these transgenic mice indicated that transgene-associated idiotype was expressed in association with endogenous Ig molecules. This association was shown to result from the formation of hybrid Ig molecules in which transgenic μ chains are associated with endogenous μ or α heavy chains.

Project Description

Major Findings:

1) CD44 expression and B cell activation.

Culture of heterogenous unprimed B cells with recombinant IL5 resulted in B cell proliferation, polyclonal Ig secretion, and phenotypic changes in the B cell population. In addition, IL5 stimulation resulted in the appearance of a B cell population which is surface Ig bright, CD44 bright, B220 (CD45) dull, and Ia dull. This population represented approximately 20% of activated B cells. When isolated on the basis of CD44 expression, this population was shown to contain nearly all of the proliferative and Ig secretory activity of IL5 activated B cell population. In vivo activation of B cells by specific antigen challenge or by the induction of a stimulatory graft-versus-host reaction resulted in the appearance of a similar $CD44^{hi}$ population. Since evidence has suggested that CD44 can function as a cell adhesion molecule, with HA as one potential ligand, the ability of resting and activated B cells to bind to (HA) was assessed. It was found that IL5-activated B cells had a uniquely increased binding to HA, and this binding was inhibited by anti-CD44. These findings suggest that CD44 expression may represent a unique marker for B cells driven to proliferation and differentiation, and that CD44 itself may function as an adhesion molecule which is involved B cell trafficking in vivo. Other B cell activating stimuli such as LPS do not induce CD44-dependent HA-binding activity. However, LPS-activated B cells demonstrate CD44-dependent HA binding immediately after exposure to a unique CD44-specific mAb, suggesting that distinct functional states of the CD44 molecule exist, perhaps reflecting differences in conformation or cytoskeletal association.

^{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.

2) Expression of CD45 on resting and activated B cells.

T cells at various stages of activation and differentiation are known to express different isoforms of cell surface CD45, reflecting in part the differential splicing of several variable exons. In contrast, B cells have generally been characterized as expressing a uniformly high molecular weight isoform of CD45. Analysis of resting and activated B cells demonstrated that activation-specific changes are induced in the expression of serologically detected CD45 epitopes. These changes can be correlated with changes detected by immunoprecipitation. In addition, a polymerase chain reaction (PCR) analysis of CD45 mRNA expression indicates that unique changes in variable exon splicing are induced by specific B cell activation stimuli.

3) Identification of new B cell activation molecules.

In an effort to identify cell surface molecules uniquely expressed during activation of B cells, a series of mAb was generated by immunizing rats with activated mouse B cells. One of the resulting mAb (GL7) reacted by flow cytometry with a subpopulation of CD3-bright thymocytes, but at only a very low level with resting peripheral T or B cells. In contrast, GL7 reacted with con A-activated $CD4^+$ 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. Another mAb generated in this fashion (GL1) reacted with activated B but not T cells. GL1 inhibited responses of $CD4^+$ T cells to activated B cells, suggesting that the target of GL1 may represent a costimulatory molecule for T cell activation.

4) Mechanism of Th cell-B cell interaction.

A highly efficient system of specific Th cell-B cell interaction was established using Ig (μ/k) transgenic B cells, which uniformly express a hapten-specific Ig receptor, and cloned antigen-specific Th cells. This cell interaction results in the specific activation and differentiation of B cells to Ab secretion. In preparation for studying early activation events during Th-B cell activation, it was established that anti-receptor antibody induced vigorous intracellular [Ca^{++}] responses in T or B cells as detected by flow cytometry.

5) Association of transgenic and endogenous Ig chains in Ig transgenic mice.

During characterization of Ig 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.

Proposed Course of Project:

1) CD44 expression and B cell activation.

The molecular basis for increased 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. Binding of radiolabeled HA to detergent solubilized B cells will be measured to analyze both the number and affinity of anti-CD44-inhibitable HA binding sites on these cells. Metabolic inhibitors will be used to determine the metabolic requirements for IL5-induced changes in CD44.

A role of CD44-mediated binding to extracellular matrix has been suggested in the in vivo trafficking of normal lymphoid cells and in the metastatic behavior of malignant cells. Preliminary experiments have demonstrated that IL5 stimulation of the murine B cell lymphoma BCL1 induces dramatically increased HA binding by these cells. The molecular basis for this will be studied. In addition, the effect of activation and altered HA binding upon in vivo trafficking of normal B cells and lymphoma cells will be analyzed.

2) Expression of CD45 on resting and activated B cells.

PCR analysis will be used to characterize further the regulation of CD45 expression in resting and activated T and B cells. Changes in alternative splicing of CD45 mRNA have been induced in monoclonal B cell lines by activation. Preliminary data indicate that the expression of lower molecular weight CD45 mRNA, in which variable exons are spliced out, is dependent upon active protein synthesis.

3) Identification of new B cell activation molecules.

Further functional characterization of the activation molecules recognized by GL7 and GL1 will be carried out. Collaborative studies have been initiated with Peter Linsley (Bristol-Meyers-Squibb) in which identification of the GL7 and GL1 target molecules will be attempted by screening of an expression library constructed from activated B cell cDNA.

4) Mechanism of Th cell-B cell interaction.

The antigen-specific interaction of Th cells and B cells will be analyzed by a digital imaging system in which intracellular $[Ca^{++}]$ can be analyzed over time in individual Th/B cell conjugates. In this system, the ability of cell interactions to signal each of these populations will be analyzed. Subsequently, the role of antigen-specific and non-specific cell interaction molecules will by analyzed by testing the effects of mAb specific for such molecules.

Publications:

Guy R, Ullrich SJ, Foo-Phillips M, Hathcock KS, Appella E and Hodes RJ. Antigen specific, MHC restricted help mediated by cell-free T cell receptor. In Gallin J and Fauci A. (Eds.): Advances in host immune defense mechanisms, Vol 7, Raven Press, New York, 1990;133-40.

Murakami S, Miyake K, Kincade PW, Hodes RJ. The functional role of CD44 (Pgp-1) on activated B cells. Immunol Res 1991;10:15-27.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09267-10 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
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 Associate	EIB, NCI
	J. Berzofsky	Senior Investigator	MB, NCI
	P. Pizzo	Chief	PB, NCI
	Y. Yarchoan	Senior Investigator	COP, NCI
	S. Broder	Director	NCI
	E. Roilides	Visiting Fellow	PB, NCI
	M. Blaese	Senior Investigator	MB, NCI

COOPERATING UNITS *(if any)*
Craig Hendrix, HIV Unit, Lackland AFB, TX; A. Landay, Rush Med. Ctr., Chicago, IL
J. Giorgi, Dept. of Immunol., UCLA Med. Sch., Los Angeles, CA.

LAB/BRANCH
Experimental Immunology Branch

SECTION
Cell Mediated Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

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☐ (a) Human subjects ☐ (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.)*

Peripheral blood leukocytes (PBL) from asymptomatic, HIV-seropositive (HIV^+) individuals exhibit a spectrum of T helper cell (Th) functional defects that is progressive and predictive for the onset of AIDS. These defects are reversed in patients who receive therapy with AZT, ddI, growth hormone, and recombinant CD4-IgG. The progression of these defects is seen "AIDS progressors", but not in "AIDS non-progressors" during a four-year follow-up. HIV-specific TH responses are detected in several groups of exposed, seronegative individuals, including homosexual men, IV drug abusers, newborns of HIV^+ mothers, an accidentally-exposed health care workers. Many of these HIV Th-reative individuals have not seroconverted nor developed AIDS symptoms on follow-up, which raises the possibility of protective TH immunity. Such protective immunity may be associated with Th1 cells, which produce IL-2 and, IFN-γ, in contrast to Th2 cells, which produce IL-4 and IL-10, and which we found to be associated with progression to AIDS.

Studies of patients with primary immune deficiencies exhibit defects in Th and APC function. Patients with Wiskott-Aldrich Syndrome exhibit a defect that involves an unstable immunogenic complex that is formed by self HLA class I, β2-microglobulin and antigenic peptide.

Project Description

Major Findings:

We have previously reported that HIV^+ individuals can be subdivided into four functionally distinct Th groups: those who respond to (1) recall antigens, (2) HLA alloantigens and (3) PHA (+++); those who respond to (2) and (3) but not (1) (-++); those who respond to (3) only (-++); and those who do not respond to any of these stimuli (---). These defects are progressive and predictive for the onset of AIDS, and we have found that a decline in these Th patterns is associated with individuals who progress rapidly to AIDS ("AIDS progressors"), whereas lack of decline in these Th parameters is associated with individuals who do not rapidly progress to AIDS ("AIDS non-progressors").

We observed reversals in these patterns of Th dysfunction in approximately 50% of AIDS patients who received AZT, ddI, or growth hormone therapy, and in 90% of patients who received recombinant CD4-IgG therapy. These results suggest that such Th analysis can be useful as a T cell functional marker of efficacy of AIDS drugs.

We have analyzed the HIV-specific Th responses of several groups of individuals who are at risk for AIDS and who have been exposed to HIV, but who have no evidence of infection. We found several such individuals from homosexual men, IV drug abusers, newborn, infants of infected mothers, and accidentally-exposed health care workers. Approximately 40% of some 60 seronegative homosexual men and IV drug abusers tested thus far showed evidence of previous exposure to HIV by our Th test. All four of the health care workers tested exhibited evidence of HIV. These results indicate that individuals can be exposed to HIV without showing evidence of infection, and raise the possibility of protective T cell immunity.

None of the 13/21 newborns showed evidence of HIV infection in repeated follow-up tests for up to one year after birth. In contrast three of the babies among the 8/21 newborns who did not exhibit HIV-specific Th immunity were HIV infected. These results demonstrate that the fetal immune system can be sufficiently mature to respond to HIV, and that immunologic exposure can occur in utero, observations again raise the possibility of protective T cell immunity against HIV infection.

Th changes in Th function that we observe appear to be associated with changes in Th1 and Th2 cytokine production, such that +++ individuals produce mainly IL-2, -++ individuals make IL- 4 but not IL-2 or IL-10, --+ individuals produce IL-10, but not IL-2 or IL-4, and --- patients appear to produce none of the above cytokines. These findings raise the possibility that Th1-Th2 cytokine cross-regulation plays are important role in resistance and susceptibility to HIV infection and/or progression to AIDS.

As an extension of our experience in AIDS immunology, we have begun to investigate Th and APC function in primary immune deficiencies. We have observed both Th and APC defects. Of particular interest is our finding that EBV-transformed B cell lines from patients with Wiskott-Aldrich Syndrome (WAS) do not serve as targets for presentation of peptide antigens in association with self HLA class I determinants at 37°C. The defect is not observed if peptides are presented in association with self class II, or if peptides are presented in association with class I at 28°C instead of 37°C, or in an excess of β2-microglobulin at 37°C. These results suggest a defect in the stability of the immunogenic complex formed by self class I; β2-microglobulin and peptide in WAS patients, and provide a useful human model for investigating antigen presentation.

Publications:

Clerici M, Tacket CO, Via CS, Muluk SC, Berzofsky JA, Shearer GM. Immunization with subunit HIV vaccine generates stronger T helper cell immunity than natural infection. Eur J Immunol 1991;

Fuchs D, Shearer GM, Boswell RN, Clerici M, Reibnegger G, Werner ER, Zajac RA Wachter HW. Negative correlation between blood cell counts and serum neopterin concentration in patients with human immunodeficiency virus type 1 infection. AIDS 1991;5:209-212.

Lucey DR, McGuire SA, Clerici M, Hall K, Benton J, Butzin CA, Ward WW, Shearer GM, Boswell RN. Comparison of spinal fluid beta2 microglobulin levels with $CD4^+$ T cell function and spinal fluid IgG parameters in 163 neurologically normal persons infected with human immunodeficiency virus (HIV-1). J Infect Diseases 1991;164:178-182.

Clerici M, Shearer GM. Cellular Immunology of HIV infection. Clinical Immunology Newsletter 1991;65:72-78.

Clerici M, Berzofsky JA, Shearer GM, Tacket CO. Exposure to HIV-1 indicated by HIV-specific T helper cell responses before detection of infection by polymerase chain reaction and serum antibodies. J Infect Dis 1991;164:178-182.

Clerici M, Tacket CO, Via CS, Muluk SC, Berzofsky JA and Shearer GM. Immunization with subunit HIV vaccine generates stronger T helper cell immunity than natural infection. Eur J Immunol 1991;21:1345-1349.

Berzofsky JA, Pendleton CD, Clerici M, Ahlers J, Lucey DR, Putney SD and Shearer GM. Construction of peptides encompassing multideterminant clusters of HIV envelope to induce in vitro T cell responses in mice and humans of multiple MHC types. J Clin Invest 1991;88:876-884.

Lucey DR, Melcher GP, Hendrix CW, Zajac RA, Goetz DW, Butzin CA, Clerici M, Warner RD, Abbadessa S, Hall S, Jose R, Woolford B, Miller S, Stocks NI, Salinas CM, Wolfe WH, Shearer GM and Boswell RN. Human immunodeficiency infection in the

US Air Force: Seroconversion, clinical staging and assessment of a T helper cell functional assay to predict change in $CD4^+$ T cell counts. J Infect Diseases 1991;164:631-637.

Berzofsky JA, Pendleton CD, Clerici M, Ahlers J, Lucey DP, Putney SD and Shearer GM. Peptides containing multideterminant clusters of human immunodeficiency virus envelope induce murine and human T cells responses in diverse histocompatibility types. Transactions of the Association of American Physicians 1991;104:64-77.

DeGroot AS, Clerici M, Hosmalin A, Hughes SH, Barnd D, Houghten R, Shearer GM and Berzofsky JA. Identification of T-helper epitodes in HIV-1 reverse transciptase: correlation with a CTL epitope. J Infect Disease 1991;164:1058-1065.

Clerici M, Shearer GM. Immunita'Cell ulomediata nell lifegione con HIV. Argomehti M' Oncologia 1991;8:50-64.

Clerici M, Giorgi JV, Gudeman VK, Chou C -C, Zack JA, Nishanian PG, Dudley Jp, Berzofsky JA and Shearer GM: Specific T-helper cell immunity to HIV-1 envelope peptides in seronegative individuals with recent exposure to HIV-1. J Infect Dis 1992;165:6-13.

Clerici M, DePalma L, Roilides E, Baker R and 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, In press.

Shearer GM and Clerici M. Abnormalities of immune regulation in HIV infection. Pediatr Res, In press.

Sher A, Gazzinelli RT, Oswald I, Clerici M, Kullberg M, Pearce EJ, Berzofsky JA, Mosmann TR, James SL, Morse HC and Shearer GM. Role of T-cell derived cytokines in the down-regulation of immune responses in parasitic and retroviral infection. Adv Immunol, In press.

Clerici M, Landay AL, Kessler HA, Venzon DJ, Lucey DR and Shearer GM. Reconstitution of T helper cell function following zidovudine therapy in HIV-infected patients. J Infect Dis, In press.

Shearer GM and Clerici M. How HIV ravages in the immune system. Current Opinion in Immunology, In press.

Shearer GM and Clerici M. T helper cell immunedysfunction in asymptomatic. HIV-1 seropositive individuals: The role of TH1-TH2 cross-regulation. Progress in Chemical Immunology. In press.

Clerici M, Roilides E, Via CS, Pizzo P and Shearer GM. A factor from CD8 cells of human immunodeficiency virus (HIV)-infected patients suppresses HLA self-restricted T helper cell responses. Proc Natl Acad Sci U.S.A. In press.

Clerici M, Roilides E, Butler KM, DePalma L, Venzon D, Shearer GM, and Pizzo PA. Changes in T helper cell function in human immunodeficiency virus infected children during dideoxyinosine therapy as a measure of antiretroviral activity. Blood, In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09268-05 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Role of CD4 and CD8 Accessory Molecules in T Cell Function

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

PI:	Alfred Singer	Chief	EIB, NCI
Others:	David Wiest	Special Volunteer	EIB, NCI
	Kelly Kearse	IRTA Fellow	EIB, NCI
	Patricia Benveniste	Special Volunteer	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.25	1.25	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have found that T cell receptor (TCR) expression and function in developing thymocytes is actively regulated by CD4-mediated signals generated by the interaction of CD4 with Ia^+ thymic epithelium. 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*) inability of TCR cross-linking to induce intracellular calcium flux. Release from these intra-thymically generated inhibitory CD4 signals results in increased TCR expression, dephosphorylation of TCR-zeta chains, and improved TCR signaling. Further, we have found that the molecular basis for low TCR expression in developing $CD4^+CD8^+$ thymocytes is a high rate of degradation of newly synthesized TCR components, and that CD4 mediated signals regulate the TCR degradation rate in $CD4^+CD8^+$ thymocytes.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

To examine the role of CD4 signals on developing T cells, we injected neonates with anti-CD4 mAb and examined TcR expression on the developing thymocytes. Remarkably, we found that the mAb caused a 3-5 fold increase in surface expression of TcR on immature $CD4^+CD8^+$ thymocytes. In order to better study the role of CD4-mediated signals in regulating TCR expression on immature thymocytes, we developed an in vitro system to study TCR expression in double positive thymocytes. We found that physical separation of immature $CD4^+CD8^+$ thymocytes from Ia^+ thymic epithelium caused the thymocytes to spontaneously increase their expression of TCR. Furthermore, CD4 signals, induced by multivalent cross-linking of anti-CD4 mAb, mimicked the presence of thymic epithelium by inhibiting TCR expression. The mechanism of TCR inhibition in immature double positive thymocytes was the retention and degradation in the Endoplasmic Reticulum of newly synthesized and assembled TCR complexes, a process that was regulated by CD4-mediated signals. Because CD4 is associated with the tyrosine kinase p56 lck, we examined the phosphorylation status of TCR-zeta, a tyrosine kinase substrate, in developing thymocytes. Consistent with the presence of a tonic CD4 signal in immature double positive thymocytes, we found that TCR-zeta was already phosphorylated in immature thymocytes resident in the thymus, but that they spontaneously dephosphorylated upon being separated from thymic epithelium.

We also examined the ability of surface TCR complexes on immature $CD4^+CD8^+$ thymocytes to transduce signals leading to intracellular calcium moblization. We found that surface TCR complexes on "uninduced" $CD4^+CD8^+$ thymocytes that had not yet escaped from CD4-meditated inhibition signaled very poorly as measured by calcium mobilization, whereas TCR on "induced" $CD4^+CD8^+$ thymocytes that had been released from CD4-mediated inhibition mobililized calcium as well as mature T cells. The relative inability of TCR on uninduced $CD4^+CD8^+$ thymocytes to signal for intracellular calcium mobilization was a function of both their low receptor number and the phosphorylation state of their TCR-zeta chains.

Thus, CD4-mediated signals in developing double positive thymocytes induced the tyrosine phosphorylation of TCR-zeta and the retention in the Endoplasmic Reticulum of newly synthesized and assembled TCR complexes, both of which contributed to the marginal signaling ability of the surface TCR complexes these cells expressed.

Publications:

Nakayama T, Samelson LE, Nakayama Y, Munitz TI, Sheard M, June CH, Singer A. Ligand stimulated signaling events in immature $CD4^+CD8^+$ thymocytes expressing competent T Cell receptor complexes. Proc. Natl. Acad. Sci. (USA). 1991;88:9949-9953.

Nakayama T, Ueda Y, Yamada H, Shores EW, Singer A, June CH. In Vivo Calcium Elevations in Thymocytes with TCR that are Specific for Self Ligands. Science. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09273-05 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

T Cell Differentiation and Repertoire Selection

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

PI:	Alfred Singer	Chief	EIB, NCI
Others:	Elizabeth Shores	IRTA	EIB, NCI
	Joseph Roberts	Guest Researcher	EIB, NCI
	Youseke Takahama	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have examined the intra-thymic differentiation of functionally and phenotypically distinct T cell subsets as well as their interaction with thymic epithelium. Studies on thymocytes from genetically defective scid mice have suggested that TcR^+ cells play a critical role in promoting the entry of thymocytes into the CD4/CD8 differentiation pathway as well as in promoting the maturation and organization of thymic medullary epithelium. Phenotypic studies on developing thymocytes have identified two distinct, but inter-related subsets of thymocytes that express identically skewed TCR repertoires, namely $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes and $Ly6C^+$ thymocytes. $Ly6C^+$ thymocytes were found to represent a readily identifiably subpopulation within each CD4/CD8 thymocyte subset; nevertheless, the $Ly6C^+$ thymocytes within each CD4/CD8 thymocyte subset expressed a distinctive TCR repertoire marked by overexpression of $V\beta8$ and expression of autoreactive TCR. Finally, we found that thymocytes readily acquire surface CD4 and CD8 determinants from other thymocytes, demonstrating that caution is necessary in using low level CD4/CD8 expression to identify novel thymocyte subsets.

PHS 6040 (Rev 1/84)

U S GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

In order to examine the general relationship between TcR expression and T cell differentiation, we have examined a genetically defective mouse strain. Mice with severe combined immune deficiency (scid), lack both receptor bearing T cells and receptor bearing B cells. It is thought that this genetic defect results from a deficiency in the recombinase enzymes necessary for receptor gene rearrangements, making it very difficult for the lymphocytes in these animals to express any antigen receptors. As a result, these animals represent an excellent model for examining how far T cell differentiation can progress in the absence of TcR expression. In fact, we found that $Thy1^+$ thymocytes from most scid mice contain only $CD4^-CD8^-$ (double negative) TcR^- cells. These cells are $IL\text{-}2R^+$ and Lyl dull, and so are similar to double negative cells from the thymi of normal mice. However, upon the introduction of TCR^+ cells into the thymi of scid mice, we found that the scid thymocytes became $CD4^+CD8^+$ even though they remained TCR^-. Thus, intra-thymic TcR^+ cells were able to promote the differentiation of TcR^- thymocytes into CD4/CD8 expressing cells. By immunohistologic examination of the thymic stroma in scid mice, we found that thymic medullary epithelium failed to organize and mature in the absence of TCR^+ cells. However, introduction of TCR^+ cells into the scid thymus induced the normal maturation and organization of thymic medullary epithelium. Thus, these studies emphasize the importance of reciprocal interactions between thymocytes and thymic stroma in T cell and thymus development.

We have characterized two minor thymocyte subpopulations, one characterized as $CD4^-CD8^-TCR\alpha\beta^+$ and one characterized as $Ly\text{-}6C^+$. Interestingly, both subsets appear late in ontogeny and have identically skewed TCR repertoires characterized by over-expression of $V\beta8$ and expression of autoreactive TCR. However, the two subsets are clearly not identical, as $Ly6C^+$ thymocytes represent a readily identifiable subpopulation within each CD4/CD8 thymocyte subset, including the mature $CD4^+$ single positive thymocyte subset. Nevertheless, the $Ly\text{-}6C^+$ thymocytes within the $CD4^+$ thymocyte subset express a skewed TCR repertoire that is identical to that of other $Ly\text{-}6C^+$ thymocytes but that is markedly discordant with the TCR repertoire that is expressed by other mature $CD4^+$ thymocytes. By a variety of criteria, $Ly\text{-}6C^+$ thymocytes appear to represent the immediate precursors of $CD4^-CD8^-TCR\alpha\beta^+$ thymocytes, but appear to be unrelated to the $Ly\text{-}6C^+$ T cells that are present in the periphery.

Finally, we have examined the expression of CD4 and CD8 determinants on the surface of $CD4^-CD8^-$ thymocytes. Surprisingly, we found that thymocytes passively acquire both CD4 and CD8 determinants from other $CD4^+$ and $CD8^+$ cells in their environment. The passive acquisition of CD8 was found to occur rapidly and to be promoted by cell surface class I MHC molecules. We think the major relevance of this observation is as an important note of caution in interpreting the significance of low level CD4/CD8 expression on developing thymocytes.

Publications:

Shores EW, van Ewijk W, Singer A. Disorganization and restoration of thymic medullary epithelial cells in T cell receptor$^-$ SCID mice: Evidence that receptor bearing lymphocytes influence maturation of the thymic microenvironment. Eur J Immunol 1991;21:1657-1661.

Takahama Y, Sharrow SO, Singer A. Expression of an unusual T cell receptor (TCR) Vβ repertoire by LY-6C$^+$ subpopulations of CD4$^+$ and/or CD8$^+$ thymocytes. Evidence for a developmental relationship between CD4/CD8 positive Ly-6C$^+$ thymocytes and CD4$^-$CD8$^-$TCR$\alpha\beta^+$ thymocytes. J Immunol 1991;147:2883-2891.

Roberts JL, Abe R, Shores EW, Singer A. Expression of Mls determinants in mice exhibiting the severe combined immunodeficiency (*scid*) mutation or X-linked immunodeficiency (*xid*) defect. J Immunol In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09275-05 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

In Vivo Study of MHC-Specific T Cells

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

PI: Alfred Singer Chief EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project has attempted to apply our understanding of the cellular mechanisms involved in in vitro anti-MHC responses to in vivo transplantation responses. In studying skin allograft rejection, we have identified the phenotype, specificity, and interaction capabilities of the T cells able to initiate and effect in vivo rejection responses. We found that in vivo exposure of effector cells to skin allografts under conditions in which T-helper cells were not activated resulted in the inactivation of the effector cells and longterm retention of the skin allograft. We found that rejection across a class I MHC barrier could occur in mice depleted of $CD8^+$ T cells by in vivo administration of anti-CD8 mAb, but that the in vivo effector cells were a novel population of anti-CD8 resistant $CD8^+$ T cells that had down-modulated their CD8 surface expression and were highly resistant to anti-CD8 blockade of their cytolytic function. In addition, we have demonstrated that rejection of skin allografvts across a class II MHC barrier requires the production of endogenous IFNgamma, presumably to induce class II expression on all the cells of the graft and make them recognizable by class II allospecific effector cells. Finally, we have assessed the cellular mechanisms mediating the rejection of fetal pancreas and Islet cell allografts.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

We have been studying transplantation immunity as the in vivo analog of the anti-MHC responses we had been examining in vitro. Our previous studies demonstrated that in vivo rejection responses to skin allografts, once initiated by antigen-specific Th cells, are mediated by antigen-specific Tk cells that assess individual cells in the dermis of the graft for expression of foreign histocompatibility antigens. The in vivo Tk cells are $CD8^+$ in the case of class I MHC alloantigens and $CD4^+$ in the case of class II MHC alloantigens. Indeed, somewhat surprisingly, we found that skin graft rejection by isolated populations of class II allospecific $CD4^+$ T-effector cells also required recognition of each cell in the graft, with rejection of only those cells expressing the foreign antigen. For keratinocytes to be recognized by $CD4^+$ effector cells, they would need to be induced to express MHC class II determinants, most likely by endogenously secreted Interferon-γ. Indeed, we found that rejection of MHC class II disparate grafts was blocked specifically by antibodies against IFN-γ, whereas rejection of MHC class I disparate grafts were not.

The concept that rejection of skin allografts was mediated by antigen-specific TK cells has been challenged by the observation that rejection of skin allografts across a class I MHC barrier still occurred in mice depleted of $CD8^+$ T cells by in vivo administration of anti-CD8 mAb. We found that such rejection did occur, but only in strain combinations in which additional histocompatibility differences were also present. We found that the cellular basis for allgraft rejection in these cases was the generation of a novel population of $CD8^+$ Tk cells that were resistant to anti-CD8 blockade, had down-modulated their CD8 expression and so were refractory to in vivo clearance by anti-CD8 mAb, and were strictly dependent upon T-helper cells specific for the additional histocompatibility antigens that were expressed on the class I disparate skin graft.

To examine the general applicability of these conclusions, we have also examined the cellular basis by which fetal pancreas and islet allografts are rejected. We found that the cellular mechanisms involved in the rejection of fetal pancreas allografts were identical to those involved in skin allografts. Furthermore, we found that fetal pancreas allografts did not undergo graft adaptation despite a 9 month residence in an immunoincompetent host. In contrast, we found that rejection of isolated islet cell allografts was solely dependent on $CD4^+$ T cells. This finding raises a number of issues that will need to be clarified in the future, including how $CD4^+$ T cells reject cells that lack MHC class II expression, and why $CD8^+$ T cells are unable to reject Islet cell allografts.

We have attempted to utilize our understanding of the basic mechanisms of allograft rejection to induce clonal tolerance. Indeed, we found that exposure of effector T cells to the Qa-1 alloantigen, in the absence of T-helper cell activation, led to clonal inactivation and long term transplantation tolerance.

Publications:

Stein PH, Singer A. Similar costimulation requirements of $CD4^+$ and $CD8^+$ primary T-helper cells: Role of IL1 and IL6 in inducing IL2 secretion and subsequent proliferation. Intl. Immunol. 1992;4:327-335.

Rosenberg AS, Singer A. Cellular basis of skin allograft rejection: an in vivo model of immune mediated tissue destruction. Ann. Rev. Immunol. 1992;10:333-358.

Stein PH, Rees MA, Singer A. Reconstitution of (BALB/c x B6)F_1 Normal Mice with Stem Cells and Thymus from Non-Obese Diabetic (NOD) Mice Results in Autoimmune Insulitis of the Normal Hosts' Pancreas. Transplantation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09279-07 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Expression of MHC Class I Genes

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

P.I.:	Dinah Singer	Section Chief	EIB, NCI
Others:	Jocelyn Weissman	Chemist	EIB, NCI
	Lisa Palmer	IRTA	EIB, NCI
	Kevin Howcroft	Staff Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION
Molecular Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

MHC class I genes encoding transplantation antigens are ubiquitously expressed, although their level of expression varies among tissues. Analysis of the 5′ flanking DNA sequence of a swine class I gene has demonstrated that in addition to the canonical promoter, this 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 homeostatic, tissue-specific levels of MHC class I gene expression. Introduction into transgenic mice of a series of nested deletion mutants which differ in the extent of the regulatory domain, reveals that the enhancer activity predominates in lymphoid tissues, but not in lymphoid tissues. The tissue-specific domain forms distinct enhancer and silencer associated complexes with cellular trans acting factors. Analysis of binding activity from a variety of cell lines and tissues reveals that enhancer binding activity is present in all extracts, independent of levels of class I expression. In contrast, the level of silencer binding activity is inversely proportional to the level of class I gene expression. These studies have led to the 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 30 kD; the silencer factor complex is approximately 95 kD.

A negative regulatory element has been identified which functions in transgenic animals in all tissues examined. Removal of this element results in markedly elevated levels of class I expression, both in transient transfection assays and in transgenic mice. Characterization of this element reveals that it is a TRE-like element. Indeed, c-jun, which binds to this element, acts as a specific negative regulator of MHC class I expression.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

Expression of individual MHC class I genes is actively regulated: large differences in the levels of class I gene expression are observed among tissues. Thus, expression is high in lymphoid tissues, but low in other tissues such as kidney and liver. However, even among the lymphoid tissues, there are distinct differences in the level of expression, such that B cells express twice as much class I as do T cells. In earlier studies, we demonstrated that introduction of one of the swine class I genes, 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. That this tissue-specific regulatory domain functions in vivo has been demonstrated through the analysis of transgenic mice generated using a variety of deletion constructs either containing or deleted of the domain. The enhancer was observed to function in lymphoid tissues, but not in non-lymphoid tissues. The patterns of expression of the transgene in non-lymphoid tissues was consistent with the activity of the silencer. Biochemical analysis of the enhancer and silencer binding factors has revealed that each is composed of two subunits; it is likely that one of the subunits is shared between the two factors. None of the subunits is capable of binding DNA independently. Both silencer and enhancer factors are redox sensitive, as evidenced by the observation that treatment with either diamide or NEM results in loss of activity. The enhancer factor is a 30 kD complex; the silencer factor is a
95 kD complex.

A second regulatory element maps between -418 and -447 bp upstream of transcriptional initiation. This element functions as a negative regulator of MHC class I gene expression. In the presence of this element, transcription from downstream promoters, either homologous or heterologous, is reduced 3-4 fold. Contained within this region is a TRE-like DNA sequence element. TRE elements are the recognition sites for the transcription factor, AP1. We have
now shown that c-jun binds to this site and functions as a transcriptional silencer of class I expression. Thus, cotransfection of a c-jun reporter gene with a class I promoter construct containing the TRE-like element results in a 3-4 fold decrease in promoter activity. Stable introduction of the c-jun expression vector into L cells causes a 10-15 fold decrease in endogenous class I gene expression. DNAse foot-printing studies confirm the binding of c-jun to the TRE-like element. In vivo, this element functions as a silencer since in tissues from transgenic mice there is greater transgene expression in the absence of the element that in its presence.

Publications:

Maguire JE, Frels WI, Richardson JC, Weissman JD, Singer DS. In Vivo Function of Regulatory DNA Sequence Elements of an MHC Class I Gene. Molec Cell Biol, 1992; in press.

Weissman JD, Singer DS. A Complex Regulatory DNA Element Associated with an MHC Class I Gene Consists of Both a Silencer and Enhancer. Mol Cell Biol 1991;11: 4217-4227.

Weissman JD, Singer, DS. Striking Similarities between the Regulatory Mechanisms Governing Yeast Mating Type Genes and Mammalian Major Histocompatibility Complex Genes. Mol Cell Biol 1991;11:4228-4234.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09281-06 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Receptor Mediated T Cell Activation

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

PI:	R. J. Hodes	Section Chief	EIB, NCI
Others:	K. S. Hathcock	Chemist	EIB, NCI
	M. Okajima	Biotechnology Fellow	EIB, NCI
	R. Abe	Visiting Associate	EIB, NCI

COOPERATING UNITS *(if any)*

Naval Medical Research Institute
Food and Drug Administration

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immune Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The effect of prior activation history on subsequent responses of cloned T helper 1 (Th1) cells to TCR-mediated stimuli was exaimed. 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 responses to anti-CD3 antibody. However, anti-CD3 induced strong phosphatidyl inosotol (PI) hydrolysis and increased $[Ca^{++}]i$ only in cells that had been maintained by stimulation with specific antigen + APC gave neither PI nor Ca^{++} responses. THe signaling pathways utilizing by Th1 cells were thus 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. It was found that both PI hydolysis 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 $[Ca^{++}]i$, demonstrated that immature thymocytes, prior to negative selection, respond specifically to self superantigen.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Major Findings:

1) Activation of Naive T Cells.

Peripheral and thymic mouse T cell populations were characterized for their expression of multiple cell surface molecules. In particular, CD45-specific mAb were used, including mAb specific for epitopes which are dependent upon expression of CD45 variable exon A or B, and an exon C-specific mAb recently developed in this laboratory. It was shown that the exon-specific epitopes detected by these mAb were expressed in non-identical distributions in peripheral and thymic populations. In the thymus, those cells which stain most brightly with antibodies to CD45 exons B and C were found to constitute a unique population that express an intermediate level of TCR. TCR cross-linking with biotinylated anti-TCR mAb induced increased intracellular [Ca^{++}] in mature peripheral T cells and in $CD4^{+}8^{-}$ and $CD4^{-}8^{+}$ thymocytes, and lesser responses in $CD4^{+}8^{+}$ thymocytes. Co-cross-linking of CD45 and TCR with biotinylated mAb resulted in profound inhibition of Ca^{++} responses by peripheral T cells, but had much less effect on the responses of thymic T cells. These results suggest that the functional coupling of CD45 to the TCR may differ in T cell subsets.

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.

The effect of TCR signalling on responsiveness to IL2 was also examined by stimulating Th1 and Th2 clones with IL2 in the presence or absence of titrated amounts of immobilized anti-CD3 or anti-TCR mAb. In most clones, regardless of Th1 or Th2 type, simultaneous stimulation with anti-CD3 mAb resulted in a marked inhibition of the proliferative response to IL2. Scatchard analysis revealed that this inhibition was not due solely to decreased expression of high affinity IL2 receptors.

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) Activation of cloned T Cells.

The molecular basis underlying differences in Ca^{++} and PI responses in cloned T cells will be investigated. The effect of cell permeabilization upon PI hydrolysis in response to TCR cross-linking will be studied in order to probe for possible roles of inhibitory intracellular mediators. cDNA probes for different phospholipase C genes will be used to study the expression of these genes in cloned T cells giving high or suppressed Ca^{++} and PI responses. These studies will be extended to a panel of Th1 and Th2 clones.

3) Signal transduction in T cell responses to endogenous superantigen.

The ability to measure specific conjugate formation and $[Ca^{++}]i$ responses by individual T cells will be applied to studies of immature T cell populations during intrathymic development, to anergic T cells generated in transgenic and bone marrow chimeric animals, and to retrovirus-infected immunodeficient mice. If early $[Ca^{++}]i$ responses are abnormal in any of these instances, the nature of the proximal defect will be analyzed by structural assessment of the TCR as well as by measurement of early phosphorylation events. If normal $[Ca^{++}]i$ responses are observed, later activation events will be analyzed to determine the nature of response defects in these cells.

Publications:

Hathcock KS, Laszlo G, Dickler HB, Sharrow SO, Johnson P, Trowbridge IS, Hodes RJ. Expression of variable exon A, B, and C-specific CD45 determinants on peripheral and thymic T cell populations. J Immunol 1992;148:19-28.

Abe R, Ishida Y, Yui K, Katsumata M, Chused TM. T cell-receptor mediated recognition of self ligand induces signaling in immature thymocytes prior to negative selection. J Exp Med 1992:in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09282-06 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Murine and Human Autoimmunity

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:	B. Bermas	Medical Staff Fellow	EIB, NCI
	E. Mozes	Guest Worker	EIB, NCI
	S. Sugihara	Visiting Fellow	EIB, NCI
	A. Rosenberg	Senior Investigator	FDA

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 MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.2	0.3

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Thyroid epithelial cell-specific $CD4^+$ and $CD8^+$ T cell lines were isolated from thyroid infiltrates of mice undergoing experimental-induced autoimmune thyroiditis. Thyroid-specific cell lines and clones proliferated and generated IL-2 in response to thyroid antigens and exhibited cytotoxic T lymphocyte (CTL) activity that is thyroid-specific and H-2 class I self restricted. These cells are TCR $\alpha\beta$+ and exhibit a broad spectrum of Vβ usage.

Patients with systemic lupus erythrematosus (SLE) exhibited both T helper cell (Th) and antigen presenting cell (APC) defects. The Th defects were complex in that different patients were identified whose peripheral blood leukocytes (PBL) were: 1) functionally intact; 2) unresponsive to recall antigens but responsive to HLA alloantigens but responsive to HLA alloantigens (ALLO) and phytohemmagglutinin (PHA); 3) unresponsive to recall antigens and ALLO, but responsive to PHA; and d) unresponsive to all of these stimulus. Approximately 80% of SLE patients also exhibit a defect in APC function. The Th defects were associated with a decrease in IL-2 production, but an increase in IL-4 production, and in some patients, an increase in IL-10 production.

Project Description

Major Findings:

Experimental autoimmune thyroiditis was induced in thymectomized, lethally irradiated B6C3F1 mice by the intravenous injection of syngeneic bone marrow and T cells depleted of CD5-bright cells. Thyroid-infiltrating lymphocytes were isolated and long-term T cell lines were established. These lines were cloned, and clones were identified that proliferated and produced IL-2 in response to thyroid antigens. Both $CD4^+$ and $CD8^+$ clones were generated. Some clones recognized antigens shared by murine and bovine thyroids, whereas others appeared to be species-specific. Clones were also isolated that exhibited thyroid-specific CTL activities that were restricted to $H\text{-}2K^k$ and $H\text{-}2D^b$. TCR-$\alpha\beta$ analyses indicated a broad spectrum of Vβ usage, with $V\beta_6$, Vβ8.1, and Vβ13 representing major components.

The intervenous injection of B6D2F1 mice with DBA/2 T lymphocytes induces a graft-versus-host reaction (GVH) that results in systemic lupus erythrematosus (SLE). Previous studies were performed using male mice. The experiments, recently repeated in female mice, result in more severe and progressive autoimmune disease than in male mice. Thus, as in human SLE, this murine GVH-induced model of SLE appears to be more prevalent in females than in males. Attempts to determine whether this prevalence in females was due to the attacking lymphocytes or to host factors was inconclusive, due to HY-associated resistance of female hosts to male donor T cells.

The human SLE-associated idiotype, 16/6, can induce SLE in susceptible (but not in resistant) strains of mice. We injected 16/6 into susceptible mice and followed the changes in Th regulation as a function of time. Soon after injection of 16/6, the mice produce elevated levels of IL-2. This is followed by a decline in IL-2 production to below control levels, which is associated with an increase in IL-4 production. This latter stage occurs at the same time as symptoms of SLE develop. The parallels between the immunedysregulatory events that occur in 16/6-induced SLE in mice and the SLE observed in humans is remarkable.

Because C57BL/6 and DBA/2 mice are respectively resistant and susceptible to SLE induced by 16/6, BXD recombinant inbred (RI) mice were studied for their resistance and susceptibility to 16/6-induced SLE, and were compared with the BXD RI mice that induce or fail to induce an SLE-like graft-versus-host reaction when their T cells are injected into $B6D2F_1$ mice. We have found that the RI strains that are resistant and susceptible to SLE by 16/6 are the same RI strains that induce or fail to induce SLE by GVH in the F_1 mice. By more extensive genetic mapping, we may be able to map a murine gene for susceptibility to lupus.

A detailed study of T helper cell function in humans with SLE has been initiated using more than 150 patients in different stages of disease. Similar

to our earlier findings in asymptomatic HIV-positive patients, we observed four distinct patterns of function: 1) patients who responded to all stimuli; 2) patients who were selectively deficient in response to recall antigens, and to HLA alloantigens, presented by autologous APC, but responsive to ALLO and PHA; 3) patients who responded to PHA only; and 4) patients who were unresponsive to all stimuli. We have also found that approximately 80% of SLE patients exhibit a defect in APC function similiar to that we observed in AIDS patients. The loss of Th function in SLE patients was assessed by T cell proliferation and IL-2 production, and was accompanied by an increase in IL-4 production, and in some patients, with an increase in IL-10. In contrast, patients who have rhematoid arithritis may exhibit dramatic increases in all three of the above cytokines, IL-2, IL-4 and IL-10.

Our study of antibodies found in the sera of SLE patients indicates that more than 50% of these patients produce anti-HIV antibodies and anti-CD4 antibodies. The potential significance of this phenomenon is under study.

Because of the parallels between the spectra of Th defects seen in SLE patients and asymptomatic HIV-infected individuals, and the findings that SLE patients make antibodies that recognize HIV antigens, the similarities in the APC defect observed in SLE patients and in patients with AIDS, approximately 70% of this project is considered to be AIDS-related research.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09285-07 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Responses of MHC Class I Genes to Exogenous Stimuli

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

P.I.:	Dinah Singer	Section Chief	EIB, NCI
Others:	Kevin Howcroft	Staff Fellow	EIB, NCI
	Adrienne Hollander	Biologist	EIB, NCI
	Lisa Palmer	IRTA	EIB, NCI
	Leonard Kohn	Senior Investigator	LBM, NIDDK
	Motoyasu Saji	Fogarty Fellow	LBM, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

Molecular Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

MHC class I genes are affected by a variety of exogenous stimuli which can either increase or decrease levels of expression. Although agents such as TNF and interferon are well known modulators of class I genes, many other factors also alter expression. We have observed that the thyroid stimulating hormone (TSH) specifically reduces transcription of endogenous class I genes in cultured 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. The TSH responsive element has been located within 135 bp upstream of the class I promoter, to a region which contains a CRE-like element. Analysis of cell extracts from normal and TSH-treated thyrocytes reveals TSH-mediated differences in the factors binding to the CRE.

Other agents are capable of modulating class I expression. Among them, insulin, hydrocortisone, and serum act as negative regulators of class I. Their sites of action are distinct from those of TSH. The DNA element responsive to serum maps to a constitutive negative regulatory element, RE-105. Analysis of RE-105 does not reveal a recognizable serum response element (SRE). However, an AP1-like binding site occurs within this region. Indeed, c-jun has been shown to be a negative regulator of class I gene expression.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

MHC class I genes are regulated both by homeostatic and non-homeostatic regulatory mechanisms. Among the known exogenous, non-homeostatic regulators are interferon and TNF. Recent studies from our laboratory have identified a number of other agents that dynamically modulate class I gene expression. Among these is the hormone, TSH. To examine the effects of TSH and other hormones on class I expression, we have studied a rat thyrocyte line, FRTL-5, which responds in culture to TSH by increasing synthesis of thyroid peroxidase, thyroglobulin, and iodide uptake. Concomitantly, TSH receptor expression declines. Thyrocytes normally express relatively low levels of class I, as does the FRTL-5 line. However, following TSH treatment, FRTL-5 expression of class I decreases even further. This decrease is evident both at the cell surface and in steady-state levels of RNA. Transcription of class I genes is reduced to about one-half to one-third the basal level following TSH treatment of the cells. This response to TSH depends on the TSH receptor (TSHr), since a variant cell line which does not express receptor does not modulate class I response to TSH. Reintroduction of TSHr by transfection of the TSHr gene restores the response. Although TSH triggers a small change in intracellular calcium, its major effect is to increase intracellular cAMP levels. Directly increasing intracellular cAMP in FRTL-5 cells by treatment with forskolin, cholera toxin or 8-bromocAMP mimics the effect of TSH. We have identified the upstream flanking sequences of the class I promoter which are responsive to the TSH effect. Using a series of 5′ deletion mutants, derived from the promoter proximal region of a class I gene, ligated to a reporter gene, were introduced into the FRTL-5 line cultured in either the presence or absence of TSH. By this approach, the responsive element was mapped to within 135 bp of the promoter. Contained within this region is a CRE-like element. Analysis by gel shift of cell extracts, reveals that the CRE is a nucleation site for DNA binding factors.

Class I expression is also regulated by a variety of other agents, including insulin, hydrocortisone and serum. All three of these agents reduce transcription of class I sequences. Using the same series of 5′ deletion mutants used to map the TSH-responsive element, the sites of action of these agents have been mapped also. They are all at sites distinct from that of the TSH element. The serum response of class I gene expression occurs not only in thyrocytes, but in a variety of other cell types as well. It has been observed that many cells (including fibroblast and lymphoblastoid cells) express markedly lower levels of class I when cultured in high serum (10-15%) than when cultured in low serum (0.5-1%). This difference is observed both at the cell surface and in steady state levels of RNA. The serum response element has been mapped to a 105 bp fragment which contains a constitutive negative regulatory element. This negative regulatory element functions both in vitro in cell lines and in vivo in transgenic animals. Both of these activities appear to map to a TRE/CRE like element. Indeed, we have shown that c-jun is a down-regulator of class I gene expression.

Publications:

Saji M, Moriarty J, Ban T, Kohn L, Singer, D. Hormonal Regulation of MHC Class I Genes in Rat Thyroid FRTL-5 Cells: TSH Induces a cAMP-mediated Decrease in Class I Expression. PNAS 1992;89:1944-1948.

Meadows G, Wallendal M, Kosugi A, Wunderlich J, Singer D. Ethanol Induces Marked Changes in Lymphocyte Populations and Natural Killer Cell Activity in Mice. Alcoholism: Clin Exptl Res 1992;16:in press.

Saji M, Moriarty J, Ban T, Singer D, Kohn L. MHC Class I Gene Expression in Rat Thyroid Cells is Regulated by Hormones, Methimazole, and Iodide, as well as Interferon. J Clin Endo Metab 1992, in press.

Kohn LD, Kosugi S, Ban T, Saji M, Ikuyama S, Giuliani C, Hidaka A, Shimura H, Akamizu T, Tahara K, Moriarty J, Singer, D. Molecular Basis for the Autoreactivity Against Thyroid Stimulating Hormone Receptor. International Immunological Reviews 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09287-05 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Marrow Graft Rejection in Allogeneic Bone Marrow Transplantation

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

PI:	Ronald E. Gress	Section Chief	EIB, NCI
Others:	Kazuhiro Kurasawa	Visiting Fellow	EIB, NCI
	Dan Fowler	Clinical Associate	MB, NCI
	Anne Husebekk	Special Volunteer	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

Transplantation Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) Minors

☐ (a2) Interviews

B,D

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

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL) may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system, CTL were cloned from the spleens of sub-lethally irradiated animals which had rejected MHC disparate marrow grafts. It was found that cloned CTL were sufficient to effect rejection of T cell depleted allogeneic marrow in lethally irradiated animals. The rejection of marrow grafts by CTL was specific for the MHC gene products expressed by the marrow cells and correlated with the cytotoxic specificity of the individual clones. Because host CTL in isolation could reject donor marrow grafts, effects on engraftment by (1) cell populations able to suppress host CTL responses, and (2) the administration of anti-CD3 monoclonal antibody in vivo, which by previous work had been shown to suppress CTL function, were studied. Cells with a specific type of suppressor activity, termed veto cells, which might suppress host rejection responses, have been reported to be present in marrow. The ability of IL-2 to enhance the activity of veto suppressor cell populations remaining in marrow after T cell depletion was investigated in vitro and in vivo. It was found that the incubation of T cell depleted marrow with IL-2 significantly increased veto activity as assessed by in vitro assays and also enhanced engraftment of MHC-mismatched, T cell depleted marrow in vivo, and that veto cells exerted their effect by clonal deletion of precursor CTL. In further studies of engraftment of T cell depleted allogeneic marrow, host mice were treated with anti-CD3 monoclonal antibody. Marked enhancement of engraftment was observed; this effect on engraftment was enduring and due to suppression of host T cell function and to the release of multiple cytokines associated with in vivo activation of T cells by anti-CD3 antibody.

U.S. GOVERNMENT PRINTING OFFICE 1991 O—564 939

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.

Cells with a specific type of suppressor activity, termed veto cells, have been reported to be present in marrow. These cells suppress those precursor CTL with specificity for antigens expressed on the surface of the veto cells. The ability of IL-2 to enhance the activity of veto suppressor cell populations remaining in marrow after T cell depletion was investigated in vitro; it was found that the incubation of T cell depleted marrow with IL-2 significantly increased veto activity. Therefore, the possibility that marrow rejection by host CTL might be suppressed by IL-2 treatment of donor marrow was evaluated. Such treatment was associated with enhanced engraftment if IL-2 was given to the host animal in addition to the treated marrow. The mechanism by which veto cells suppress CTL responses is not known. Two barriers to the study of the mechanism has been unreliable suppression of CTL responses by putative veto cell populations, and the low frequency of precursor CTL in the responder population, making it technically difficult to differentiate death of precursor CTL from induction of anergy. The first difficulty was overcome by incubation of the suppressor cell population with IL-2. Studies showed an inhibition of veto activity by antisera with specificity for cytolytic granules, indicating that lysis of precursor CTL with clonal elimination, rather than induction of clonal anergy, may be the likely mechanism for the suppression of CTL responses by IL-2 enhanced veto cells. Studies with transgenic mice, in which responder T cell populations contain precursor CTL with a defined antigen specificity at high frequency, directly demonstrated that the mechanism by which veto cell activity mediates suppression of CTL responses is by clonal deletion of precursor CTL.

With the demonstration that CTL are sufficient to effect marrow graft rejection, studies were undertaken to evaluate the possible effects of anti-CD3 treatment on marrow engraftment in a mouse model. The antibody used was

specific for the ε chain of the murine CD3-T cell receptor complex, can suppress skin graft rejection, and can cause both short term and long term in vivo T cell dysfunction. The intact antibody results in detectable T cell activation in vivo while the $F(ab')_2$ from of the antibody does not. T cell immunosuppression is pronounced at one week after administration of the intact antibody. It was found, however, that in vivo treatment with anti-CD3 administered seven days before infusion of bone marrow did not enhance engraftment of allogeneic marrow in sublethally irradiated hosts. Therefore, immunosuppression provided by treatment with anti-CD3 monoclonal antibody was not sufficient to prevent rejection of allogeneic marrow graft. Studies also demonstrated that administration of intact anti-CD3 to mice resulted in T cell activation within hours of administration (manifest by increased IL-2 receptor expression and by enhanced proliferation of spleen cells from treated animals to exogenous IL-2 in vitro). Because this activation also resulted in secretion of colony stimulating factors (CSF) detectable in the serum and was associated with extramedullary hematopoiesis in the spleen, the effect of anti-CD3 antibody administration at the time of allogeneic marrow infusion was evaluated. The injection of anti-CD3 monoclonal antibody with the donor marrow resulted in extensive allogeneic chimerism. Non-activating, anti-T cell monoclonal antibodies also facilitated engraftment, but resulted in lesser degrees of chimerism. Similar findings occurred in studies in subhuman primates. In the mouse, incubation of T cell depleted allogeneic marrow in the supernatant of spleen cells incubated with anti-CD3 antibody in vitro also resulted in enhancement of engraftment in the presence of, but not in the absence of, host T cell suppression. Therefore, the enhancement of marrow engraftment by in vivo administration of anti-CD3 monoclonal antibody appears to be due to both suppression of host T cell function and the presence of growth factors. Identification of specific factors which are present in the supernatants of spleen cells exposed to anti-CD3 monoclonal antibody, and which promote engraftment of T cell depleted, MHC-disparate marrow indicates that multiple factors are involved.

Publications:

Hirsch R, Archibald J, Gress RE. Differential T cell hyporesponsiveness induced by in vivo administration of intact or $F(ab')_2$ fragments of anti-CD3 monoclonal antibody: $F(ab')_2$ fragments induce a selective T helper dysfunction. J Immunol 1991;147:2088-2093.

Blazar BR, Hirsch R, Gress RE, Carroll SF, Vallera DA. In vivo administration of monoclonal antibodies or immunotoxins in murine recipients of allogeneic T-cell depleted marrow for promotion of engraftment. J Immunol 1991;147:1492-1503.

Berlin PJ, Bacher JD, Sharrow SO, Gonzalez C, Gress RE. Monoclonal antibodies against human T-cell adhesion molecules: Modulation of immune function in non-human primates. Transplantation 1992;53:840-848.

Herold KC, Bluestone JA, Montag AG, Parihar A, Wiegner A, Gress RE, Hirsch R. Prevention of autoimmune diabetes with a non-activating form of anti-CD3 mAb. Diabetes 1992;41:385-391.

Hiruma K, Nakamura H, Henkart PA, Gress RE. Clonal deletion of post-thymic T cells: Veto cells kill precursor cytotoxic T lymphocytes. J Exp Med 1992;175:863-868.

Hiruma K, Hirsch R, Patchen M, Bluestone JA, Gress RE. Effects of anti-CD3 monoclonal antibody on engraftment of T cell depleted bone marrow allografts in mice: Host T cell suppression, growth factors, and space. Blood 1992;79:3050-3057.

Hirsch R, Gress RE, Bluestone JA. Anti-CD3-mediated immunotherapy: A murine model. In Burlinghan, W. (ed.): Critical Evaluation of Antibody Therapies in Transplantation, pp.1-15. CRC Press, Boca Raton.

Hiruma K, Gress RE. Cyclosporine A and peripheral tolerance: Inhibition of veto cell-mediated clonal deletion of postthymic precursor cytotoxic T lymphocytes. J Immunol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09288-05 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
T Cell Function in T Cell Depleted Bone Marrow Transplantation

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

PI:	Ronald E. Gress	Section Chief	EIB, NCI
Others:	Crystal Mackall	Clinical Associate	PB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION
Transplantation Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B,D

☐ (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 demonstrated that CD4+ T cell reconstitution and development of in vivo T cell immunocompetence correlated with the number of T cells infused in the marrow raising the possibility that residual T cells in the infused T cell-depleted marrow played a central role in the generation of subsequent T cell populations. This conclusion has been confirmed in murine studies in which three T cell progenitor pools have been identified which contribute to final T cell repopu-lation following marrow transplantation. The functional capacities of these regenerated T cell populations is also of interest. The human T helper cell response to xenogenic MHC encoded antigens expressed by stimulating murine cell populations has been studied and found to be of special use in the assessment of human T helper cell function in that this primary response requires reprocessing of the stimulating murine antigens and 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 in part to a lack of murine antigen presenting cell activation.

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

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. 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 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 defect would seem to involve defects at the level of antigen presenting cell activation and initial T

cell activation. Late events in T cell activation appear to be intact as activated murine APC effectively stimulate human T cells to produce IL-2.

Publications:

Read EJ, O'Shaughnessy JA, Yu MY, Cottler-Fx M, Denikoff AM, Cowan KH, Gress RE. Flow cytometric quantitation of circulating hematopoietic progenitor cells in breast cancer patients on chemotherapy. In Gross, S., Gee, A., and Worthington-White, D.A. (eds.): Bone Marrow Purging and Processing. Wiley-Liss. New York, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09289-02 EIB

PERIOD COVERED: October 1, 1991 to September 30, 1992

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

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

PI:	D. M. Segal	Section Chief	EIB, NCI
Others:	S. Andrew	Visiting Fellow	EIB, NCI
	P. Perez	Guest Researcher	EIB, NCI
	A. George	Special Volunteer	EIB, NCI
	C. Jost	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*: Creative Biomolecules, Inc. Hopkinton, MA
James S. Houston, PI

LAB/BRANCH: Experimental Immunology Branch

SECTION: Immune Targeting Section

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

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☐ (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 project has been initiated to produce by genetic engineering, a single polypeptide chain with the antigen binding ability of a bispecific antibody. We have produced several cloned plasmids containing inserts encoding single chain Fv (sFv) molecules. One of these, an anti-DNP sFv, has been expressed in E. coli as inclusion bodies. This protein has been refolded into a molecule with antigen-binding activity.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864-939

Project Description

Major findings

Single chain bispecific antibody

Antibodies consist of 4 polypeptide chains, 2 light and 2 heavy chains of 25 and 50 kD each, respectively. Each chain is segmented into 12.5 kD domains of homology. All of the antibody binding specificity resides in the N terminal domains, known as variable or V domains, because they vary in amino acid sequence from antibody to antibody. A native antibody molecule contains 2 identical antigen binding regions, each consisting of a light chain variable domain (V_l) and a heavy chain variable domain (V_h). The V_h and V_l domains are non-covalently linked to one another, forming a distinct 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, providing that a spacer of at least 12 residues is inserted between the V_l and V_h chains. 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 prepare constructs in which two sFvs are linked together genetically, to make a single chain bispecific antibody (SCBA). Bispecific antibodies can target cytotoxic cells against tumor and virally infected cells, and can enhance antigen immunogenicity, in vivo and in vitro. Currently the methods for producing bispecific antibodies that would be suitable for clinical use are complicated, time consuming and give low yields of material. The production of SCBAs should provide an improved way of making bispecific antibodies.

mRNA was isolated from several hybridoma cell lines. By using appropriate oligonucleotide primers, DNA constructs encoding sFv proteins were generated and expanded using the PCR and cloned into Bluescript. We have generated sFv clones from OKT3 (anti-CD3), VD2 (anti-CD16), U7.6 and MOPC 315 (anti-DNP), and Z-12 (anti-influenza virus). All of the clones have been sequenced and contain open reading frames encoding for proteins that are highly homologous to other immunoglobulins from the Kabat or GenBank data bases. In addition, the clones were transcribed using the Bluescript T7 promoter and T7 polymerase, and the RNA thus produced encoded peptides of the correct molecular weight in reticulocyte lysates.

Several of our sFvs have been subcloned into the pETlld expression vector, and used to produce protein in E. coli as inclusion bodies. We have concentrated on the U7.6 construct as a test for producing active sFv. Bacteria carrying pET-U7.6 produced sFv protein upon induction with IPTG. Inclusion bodies were isolated from lysed bacteria and the contents dissolved in 6 M Gdm.Cl with reducing agent. We have tried several protein refolding procedures, and so far the best has involved diluting the denatured protein in medium containing 0.4M arginine, 3mM reduced and 5 mM oxidized glutathione, pH 8. This produces a protein that binds DNP-BSA in an ELISA (using goat anti-mouse Fab and alkaline phosphatase rabbit anti-goat as developing reagents). The protein does not bind to BSA or DNP-BSA in the presence of DNP hapten or excess anti-DNP. U7.6

sFv has been purified by affinity chromatography on a DNP-lysine-Sepharose column (using hapten elution), and gives a single band of the correct molecular weight by SDS-PAGE and by FPLC using a Superose 12 column. The yield of active protein is 1.7%.

A SCBA has been constructed using OKT3 sFv linked to U7.6 sFv through a $(Pro)_8$ spacer. Protein has been produced in inclusion bodies, and after refolding the protein binds specifically to DNP-BSA.

This project is being carried out under a cooperative research and development agreement (CRADA) with Creative Biomolecules of Hopkinton, MA.

T Cell Receptor Single Chain Fv

As an offshoot of our studies using sFv's from antibodies, we have begun constructing an sFv from a T cell receptor (TCR). We have chosen to work on the 2B4 TCR, because this receptor has well known specificity (a pigeon cytochrome C peptide bound to E^k), stable 2B4 T cell hybridomas exist, and the receptor has been cloned and sequenced. V_β and V_α cDNA clones have been isolated, and a single chain Fv constructed by linking the C terminus of V_α with the N terminus of V_β through a $(gly_4\ ser)_3$ polypeptide linker. The nucleotide sequence was verified, and the construct was subcloned into the pET11d expression vector, and protein was produced in E. coli as inclusion bodies.

The inclusion bodies were solubilized in 6M Gdm.Cl with reducing agent, and refolded by dilution in 0.4M arginine containing a redox mixture. Only small amounts of soluble material of the correct molecular weight have been recovered to date. Most of the protein aggregates covalently, probably due to two extra cys residues in the 2B4 V_β region. Nevertheless, the correctly renatured protein binds the A2B4 (anti-2B4 idiotype), KJ25 (anti-V_β3), and RR8.1 (anti-V_α11) mAbs. The A2B4 antibody is at least in part conformationally dependent because it does not bind to the reduced 2B4 sFv. We have not yet obtained enough material to test the sFv for antigen binding activity.

Proposed Course of Project

Our first milestone for this project is the development of a SCBA that can induce human T cells to specifically lyse TNP-coated target cells. We should be in a position relatively soon to test whether our first SCBA does have both activities. The most important problem facing this project is the low yields of antibody that we obtain after refolding. We will continue to alter our folding condition to improve yields, but we will also test other expression vectors. First we will try bacterial secretion systems. These systems can correctly form disulfides in the periplasmic space, and we are hoping this will improve our yield while maintaining high protein production. We are also trying yeast expression systems (in collaboration with Dr. Pilar Perez in Salamanca), and we may also try mammalian expression vectors.

With regard to the TCR sFv, we also need to improve yields before testing for activity. We will use different folding strategies and expression systems (as above), and also try removing the extra cys residues in V_β by site directed

mutagenesis. As for testing for binding activity, we will try to bind peptide-pulsed APC to immobilized 2B4 sFv, to block cytochrome C presentation to 2B4 cells with the 2B4 sFv, and to target antigen pulsed APC for lysis with a 2B4 sFv x anti-CD3 bispecific antibody. Preliminary evidence from other laboratories suggests that the 2B4 TCR binds with low affinity, and it may provedifficult to measure binding directly. Ultimate goals of this project are to use the 2B4 sFv to gain information about the affinity of the TCR/antigen interaction, to structurally characterize the receptor (optimally by X-ray crystallography), and to use the sFv in targeting studies, perhaps as part of a SCBA.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09290-02 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Targeted Antigen Presentation

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

PI: D.M. Segal Section Chief EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immune Targeting Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Bispecific antibodies can greatly enhance the ablility of an antigen to evoke an antibody response, in mice, paralleling their ability to enhance antigen processing and presentation in vitro.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description

Major Findings

We have previously shown that the binding of protein antigens to antigen presenting cells with bispecific antibodies enhances their processing and presentation to T helper cells, in vitro. These findings led to experiments using bispecific antibodies to immunize mice. Bispecific antibodies were prepared by chemically crosslinking an antibody with specificity for hen egg lysozyme (HEL) to various other antibodies, each specific for a particular APC cell surface component. Mice were then immunized with HEL in the presence or absence of bispecific antibodies and antibody production was monitored after the primary challenge and following a secondary boost. Bispecific antibodies that bound antigen to MHC class I or class II molecules, to $Fc_{\gamma}R$, but not to surface IgD, enhanced the immunogenicity of HEL. For example, anti-HEL x anti-class II bispecific antibodies decreased the amount of antigen required to elicit a primary anti-HEL antibody response in mice by 300 fold and the amount required to prime for a secondary response by 10^3-10^4 fold. Bispecific antibodies were as effective as incomplete Freund's adjuvant in generating antibody responses. Since adjuvants cannot be used in humans, bispecific antibodies could prove useful for immunizing people, especially in cases where, due to scarcity or toxicity, minute doses of antigen must be used.

Proposed course of project

We plan to renew work on this project by examining the ability of bispecific antibodies to enhance antibody responses to influenza and mouse mammary tumor viruses.

Publications:

Snider DP, Kaubisch A, Segal DM. Enhanced antigen immunogenicity induced by bispecific antibodies. J Exp Med 1990;171:1957-1963.

Snider DP, Uppenkamp IK, Titus JA, Segal DM. Processing fate of protein antigen attached to IgD or MHC molecules on normal B lymphocytes using heterocrosslinked bispecific antibodies. Molec Immunol in press

Patents

Segal DM, Snider DP US Patent 7,516,879: Bispecific antibody enhancement of immunization.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09291-03 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Genomic Organization, Characterization, and Regulation of the Human ζ Gene

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

PI:	A.M. Weissman	Senior investigator	EIB,NCI
Others:	C. Cenciarelli	Visiting fellow	EIB, NCI
	J.P. Jensen	Chemist	EIB, NCI
	B. Rellahan	IRTA Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The ζ subunit of the T cell antigen receptor is a limiting component in receptor assembly and is required for the targeting of the T cell antigen receptor to the cell surface. Zeta is differentially regulated relative to the other T cell receptor components as witnessed by its expression in natural killer cells. It is also clear that this subunit undergoes alternative splicing to yield another product that has been characterized in murine cells and termed η. We have characterized the gene encoding the human ζ subunit. The intron/exon organization of the ζ gene and the sites of transcription initiation have been determined. We have also developed this gene as a genetic marker. The cloning of the ζ gene has provided us with the tools to study the transcription of this gene. Studies are underway aimed towards the determination of the promotor and enhancer elements of the ζ gene. A region at the 3'end of the human ζ gene that is highly homologous to the murine η exon has been characterized. This has led us to study the expression of this η -like region and to determine the nucleic acid sequence of this region from a number of other mammalian species. As an extension of our findings we have also been studying the relative expression of ζ and η on a message and protein level during thymic development.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major findings:

The human ζ gene consists of 8 exons that encode the ζ cDNA. The first intron is greater than 8 kb is size with the remaining introns ranging from 0.7 to 3 kb. Ribonuclease protection studies demonstrate a large number of transcription initiation sites with the most 3' being 66 bases upstream from the translation initiating ATG. The other sites are distributed over approximately 115 bases. A series of plasmids containing regions 5' from the site of translation initiation cloned in front of the luciferase gene have been generated. These constructs are being utilized to study the transcription of the ζ gene. Initial studies suggest that there are at least two non-overlapping regions capable of initiating transcription.

The ζ gene contains a variable number tandem repeat (VNTR) restriction fragment length polymorphism (RFLP) within inton 5. We have utilized this VNTR to map ζ on human chromsome 1q. The position of this gene suggested the possibility that ζ may be linked to a family of receptors for immunoglobulin (Fcγ RII-Fcγ RII) to determine whether this was the case we have utilized a probe from this cluster to develop an RFLP and have mapped this gene family to within 10 centimorgans of ζ on chromosome 1q.

The ζ gene contains a 3' region that is highly homologous on a nucleotide level the murine η exon. However the presence of multiple insertional and deletional mutations would result in a highly divergent structure when analyzed on a protein level. This observation has led us to carefully analyze the transcription and splicing of this η - like region. Our studies indicate that the human equivalent of the murine η exon is transcribed and spliced to upstream exons of ζ at extraordinarily low levels. We have also carried out cross species analysis of the η region from a number of other mammalian species by PCR amplification and sequencing of genomic DNA. This analysis is remarkable as it demonstrates regions of nucleic acid conservation interspersed with regions of marked frame shifting. This leads to total conservation of the first 7 deduced amino acids with essentially no conservation beyond this region.

In murine cells a correlation has been established between the level of η and the ability of cells to undergo receptor mediated apoptosis. As apoptosis has been suggested as a means of negative selection in the thymus, the relative frequency of ζ and η message from thymocytes from mice of different ages was evaluated. These ages corresponded to periods when there is differential succeptibility to anti-receptor antibody mediated apoptosis. No substantial correlation between the relative levels of ζ and η in total thymus and the propensity of the thymic population at that age to undergo apoptosis was found.

Publications:

Zacharchuk CM, Mercep M, June CH, Weissman AM , Ashwell JD. Thymocyte Susceptibility to Clonal Deletion Varies During Ontogeny: Implications for Neonatal Tolerance. J Immunol 1991;147:460-465.

Jensen JP, Hou D, Ramsburg M, Taylor A, Dean M, Weissman AM. Organization of the Human T Cell Receptor ζ/η Gene and to Genetic Linkage to the Fcγ RII - Fcγ RII Gene Cluster. J Immunol 1992;148:2563-2571.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09292-03 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Signal Transduction in T lymphocytes

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

PI:	A.M. Weissman	Senior Investigator	EIB, NCI
Others:	C. Cenciarelli	Visiting Fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

(Formally Titled: "Signal Transduction in Permeabilized T Cells")

T cell receptor (TCR) mediated signal transduction involves a complex series of early events that culminate in cellular activation. The ζ subunit of the TCR has several characteristics that make it unique. It is structurally unrelated to the other invariant receptor subunits; it is expressed in natural killer cells in which other receptor components are not expressed; it undergoes tyrosine phosphorylation in response to T cell receptor activation; and has been shown to transduce signals independently of other receptor components. We are interested in understanding the molecular mechanisms whereby receptor occupancy is coupled to intracellular events. Our studies have concentrated on understanding the role that ζ plays both as an effector molecule and as a substrate for receptor activated protein tyrosine kinases. Studies are carried out primarily in the murine T lymphocyte hybridoma 2B4 and in variants of this hybridoma. Studies in 2B4 cells have been concentrated in two areas: systematic mutagenesis of the tyrosine residues of the ζ subunit; and development of a permeabilized cell system for manipulation of the intracellular environment. Studies in permeabilized cells have focused primarily on establishing whether GTP binding proteins play a role in TCR-mediated signalling.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0—864-939

Project Description

Major Findings:

Tyrosine phosphorylation of the ζ subunit results in a change in its migration under reducing conditions on SDS/PAGE from 16 kD to 21 kD. One and two dimensional analysis of phosphorylated ζ has suggested that multiple tyrosines undergo phosphorylation and that this process is cooperative in nature. To disssect which tyrosines undergo phosphorylation a systematic mutational analysis of the ζ subunit was undertaken in which each of the intracellular tyrosines was mutated to phenylalanine. Following mutation these constructs were transfected into ζ negative variants of the T cell hybridoma 2B4 and analyzed for phosphorylation and functional activity. Mutation of tyrosine 123, 142 or 153 results in marked abnormalities in the migration of phosphorylated ζ suggesting that these tyrosines are involved in a cooperative process. Mutation of tyrosine 111 results in a marked dimumition in the level of phosphorylation of ζ. However those molecules that were phosphorylated demonstrated an apparent Mr of 22,000 suggesting that they were in fact hyperphosphorylated relative to the predominant of 21 kD species found with wild type ζ. Our results suggested that all of the 6 intracelluar tyrosine of ζ could potentially serve as tyosine known substrates in-vivo. However it is apparent from our analysis that the 4 most carboxyl tyrosines are most crucial for the generation of the 21 kD form of phospho-ζ. The functional consequences of these mutations was assessed by analysis of IL-2 production. No qualitative abnormalities were detected as a result of the mutations.

To characterize the molecular events that couple receptor occupancy to intracellular signalling we have developed a permeabilized cell system using the T cell hybridoma 2B4. After adjustment of buffer conditions we have been able to determine conditions in which receptor-stimulated tyrosine phosphorylation can take place in such a system. Initial studies have focused on the effect of nucleotides in this system. Treatment of cells with the non-hydrolyzable GTP analogue GTPγS results in a dose dependent enhancement of tyrosine phosphorylated ζ. This effect can also be seen with another GTP analogue Gpp(NH)p. The specificity of this effect was established using ATP γS, App(NH)p and GDPβS. The GTP effect required receptor occupancy hence a GTP binding protein would seem to be synergizing with receptor occupancy to facilitate the phosphorylation of ζ. Further studies have established that the effect was not due to protein kinase C activation. In additions the GTP effect was not due to changes in cell surface expression of receptor. Ongoing studies are focused on determining whether other tyrosine kinase substrates are similarly affected and also towards determining the molecular mechanisms responsible for this effect.

Publications:

O'Shea JJ, Weissman AM, Kennedy ICS, Ortaldo JR. Engagement of the Natural Killer Cell IgG Fc Receptor Results in Tyrosine Phosphorylation of the ζ Chain. Proc Natl Acad Sci USA 1991;88:350-354.

Frank SJ, Cenciarelli C, Niklinska BB, Letourneur F, Ashwell JD, Weissman AM., Mutagenesis of T-Cell Receptor ζ Chain Tyrosine Residues: Effects on Tyrosine Phosphorylation and Lymphokine Production. J Biol Chem 1992, in press.

Cenciarelli C, Hohman RJ, Atkinson P, Gusovsky F, Weissman AM. Evidence for GTP Binding Protein Involvement in the Tyrosine Phosphorylation of the T Cell Receptor ζ Chain. J. Biol Chem 1992, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09296-01 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Activation- Induced Ubiquitination of the Cell Antigen Receptor

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

PI:	A. M. Weissman	Senior Investigator	EIB, NCI
Others:	C. Cenciarelli	Visiting Fellow	EIB, NCI
	D. Hou	Hughes Fellow	EIB, NCI
	J.P. Jensen	Chemist	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

T Cell Antigen Receptor Occupancy results in the ubiquitination of TCR subunits including the ζ and δ subunits. Modified subunits are associated with other receptor subunits and persist for at least 2 hours after acute activation. Ubiquitination is an active process and occurs in normal and tumor cells of both murine and human origin. Stdies are currently underway animal towards understanding in TCR Function.

PHS 6040 (Rev 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 O—864 939

Project Description

Major Findings:

Analysis of activated murine cells on two dimensional non-reducing-reducing SDS/PAGE revealed a ladder of higher molecular weights forms of the ζ molecule which could be discerned in con A blasts as well as in acutely activated thymocytes, splenocyes and hybridoma cells. Further analysis utilizing NEPHGE/PAGE gels revealed that this ladder represented derivitization of ζ with multimers of a neutral 8 kDa structure. This suggested the possibility that ζ was ubiquitinated on activation. To test this possibility, immunoprecipitates from control and anti-receptor antibody activated 2B4 cells were resolved both on diagonal gels and on two dimensional NEPHGE/PAGE. Immunoblotting was carried out with monoclonal anti-ubiquitin reagents. This analysis revealed a ladder of activation-induced species that co-migrated with the activation-induced immunoreactive forms of ζ. This indicatede that ζ was in fact ubiquitinated. We have determined that the CD3 δ subunit is also ubiquitinated.

Although TCR engagement clearly results in an increase in receptor ubiquitination, we have observed this modification at a low constitutive level in fresh spenocytes and thymocytes. Ubiqutinated ζ subunits were immunoprecipitated equally well with anti-TCR ε reagents and with anti-ζ reagents. It is therefore clear that these ubiquitinated subunits are not isolated intermediates in a pathway to degradation but are rather components of apparently intact TCRs.

To determine whether the finding of ubiquitinated ζ represented the result of an activation-induced process or a redistribution from a detergent insoluble fraction, cells and insoluble pellets were treated with DNAase and extracted with SDS. Based on these studies we conclude that we are witnessing an active process. Additionally, it is clear that this alteration occurs in normal human cells and human T cell tumor lines. Kinetic analysis demonstrated that ubiquitinated forms appear within 5 minutes, and persisted for at least 2 hours after acute activation. This process occurs in all T cell types that have been analyzed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09297-01 EIB

PERIOD COVERED

October 1, 1991 to September 30, 1992

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

Characterization of Novel Developmental Mutations Caused by Retroviral Insertion

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

PI:	Michael Kuehn	Expert	EIB, NCI
Others:	Xunlei Zhou	Visiting fellow	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5		

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

To understand mammalian developmental mechanisms at the molecular level, it is necessary to identify and isolate developmental genes. In the mouse, mutations that result in developmental abnormalities have identified a number of candidate developmental genes. However, cloning genes identified by spontaneous or chemically induced mutations is technically difficult and has been accomplished only rarely. A powerful and direct method to induce a mutation and molecularly clone the mutated gene is insertional mutagenesis. Insertional mutations result from the integration of foreign DNA into a gene. The integrated sequence serves a dual purpose. It not only creates the mutation but also acts as a molecular marker "tagging" the locus. By recloning the integrated sequence the affected gene can be easily recovered as well.

Insertional mutagenesis occurs spontaneously in the mouse due to endogenous retroviral infection of embryos or germ cells; and through experimental introduction of DNA or retrovirus to produce transgenic mice. Approximately 10% of proviral integrations or transgenic DNA insertions are into genes, resulting in mutations. We have analyzed 2 transgenic strains derived from embryonic stem (ES) cells infected with a retroviral vector for insertional mutations. These strains are unique in that they each carry multiple, independently integrated proviral sequences. The 413 strain segregates 4 proviruses and the 412 strain, 23. Systematic genetic and molecular analysis has allowed us to determine that 5 of the 27 proviruses are associated with mutations. We are now carrying out molecular and phenotypic studies on these mutants.

U.S GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Project Description

Major Findings:

Of the 5 insertional mutations under study, work is most advanced on the 413-d mutant. This recessive mutation results in embryonic death. To better understand the phenotype we have carried out a histological examination of serially sectioned embryos. These studies have shown that abnormalities are apparent by the egg cylinder stage (day 7 of embryogenesis). Mutant embryos at this stage are characterized by hyperplasia of embryonic and extra-embryonic ectoderm. Using image analysis we have determined that mutant ectodermal cells are not only present in greater numbers (two to four times that in normal embryos) but are also smaller in size (two thirds that of normal cells) at this stage. The increase in cell number and decrease in cell size in these lineages must arise from shorter cell cycle times. We estimate that a cell division rate of 2.7 hours from day 6 to day 7 of embryogenesis would be required to produce the observed number of cells. The hyperplastic embryonic ectoderm then undergoes rapid and extensive necrosis and is totally gone by day 9 of embryogenesis. Examination of these later stage mutant embryos also suggests that trophoblastic giant cells are hyperplastic as well, but the abnormal shape and size of the mutants prevents a meaningful comparison of cell number in this lineage to normal embryos. The gene identified by 413-d insertional mutation, which we have named hyperplastic ectoderm (*hec*), would appear to play an important role in the growth control of these early cell lineages.

The molecular characterization of the *hec* gene is currently underway. The 413-d provirus as well as flanking cellular sequences were cloned from a cosmid library of genomic DNA. The clones were isolated using genetic selection for the *neo* gene contained in the retroviral vector. Nine kanamycin resistant clones selected out of the library were restriction mapped and shown to overlap, with each containing an entire provirus and varying amounts of 5′ and 3′ flanking DNA. Together, the clones cover a genomic region totaling more than 60 kb around the site of the 413-d retroviral insertion. To locate and characterize the *hec* gene, we first identified regions of unique sequence DNA flanking the provirus. These regions were examined as candidate exons for the *hec* gene and also used to map the *hec* gene to chromosome 10 by restriction fragment length polymorphism analysis of inter-species backcross DNA. The unique sequence fragments were analyzed to determine if they contained transcribed sequences. However, Northern analysis of RNA prepared from undifferentiated ES cells, differentiated ES cells (both simple embryoid bodies and further differentiated cystic embryoid bodies) and staged embryos failed to detect any RNA hybridizing to these fragments. The fragments were then tested for cross-hybridization to DNA from other organisms. The probes were hybridized to "zoo" blots of human, cow, rat, vole, chicken, Drosophila and yeast DNA and one probe showed a hybridization signal with the rodent sequences after high stringency washes. To determine whether this region contains sequences representing the exon(s) of an evolutionarily conserved gene, we sequenced the fragment and looked for splice acceptor and donor sequences and open reading frame(s). This fragment indeed contained a perfect match to the splice donor

consensus sequence and has been the focus of subsequent attempts to isolate the *hec* gene. Using oligonucleotide primers derived from this region, we have carried out reverse transcription/polymerase chain reaction (RT-PCR) analysis of RNA samples and detected transcription in all embryonic cell lines looked at as well as several other cell lines. The amplified region has been used as a probe to screen cDNA libraries made from mouse embryos. Several positive clones have been recovered and are currently being characterized.

These cDNAs will provide reagents essential for determining the structure of the *hec* gene and its pattern of expression during development.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 09298-01 EIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Multiplex Method for Large Scale Insertional Mutagenesis in Transgenic Mice

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

PI:	Michael Kuehn	Expert	EIB, NCI
Others:	Linda Lowe	Biologist	EIB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5		

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Although insertional mutagenesis in transgenic mice has allowed a number of new mutants to be identified and studied, the overall insertion frequency per locus is quite low. Our findings with two transgenic strains, 412 and 413, carrying multiple transgenes (proviruses) confirm previous estimates that only 10% of integrations of transgenes lead to recessive developmental mutations. It has been estimated that the probability of generating a mutation by transgene insertion is 100 fold lower than the probability of producing a mutation using ethylnitrosourea, a potent chemical mutagen. This estimate was based on transgenic strains usually having only one site of integration of exogenous DNA. While screening animals with only one or a few insertions seems theoretically desireable, because large numbers of insertions might make it difficult to determine which one causes the mutation, the low mutagenesis frequency makes large scale insertional mutagenesis with conventional transgenic strains impractical. Although the frequency of insertional mutagenesis cannot be changed, the efficiency can be increased by screening transgenics with multiple insertions, as we have shown with the 412 and 413 strains. We have developed an approach to improve the efficiency and applicability of this method even more, and thereby make it possible to identify and clone a large number of developmentally important genes. We are now implementing this strategy.

Project description

Major findings:

We have developed an approach to follow the inheritance patterns of large numbers of proviruses, newly integrated into the germ line of a transgenic mouse strain. We have created eighteen new retroviral vectors by insertion of different fragments of phage lambda DNA into a basic retroviral vector. We have developed protocols to infect ES cells so that individual clones have from 10 to 20 copies of each of these 18 different types of vectors. Because each vector has its own unique molecular tag, each small group of 10 to 20 proviruses can be separately analyzed in ES cell clones and mice derived from infected ES cells. This "multiplex" analysis is done by carrying out multiple rounds of probing, stripping and reprobing of the same Southern transfers of DNA, with probes homologous to the specific lambda sequence carried by each vector. With 20 copies of each of these 18 retroviral vectors, it will be possible for us to examine 360 total retroviral insertions in each transgenic mouse strain made from infected ES cells for insertional mutations. Without the multiplex feature, we would be limited to only 10 to 20 insertions. In a pilot experiment, 6 individual cell lines each producing a high titer of a single retroviral vector were derived. Retroviral DNA for each of the 6 was separately transfected into the GP+AM amphotropic producer cell and the virus subsequently shed by these cells was used to infect the GP+E88 ecotropic cell line. Ecotropic cells were immediately put under G418 selection to kill off uninfected cells. This procedure was found to give a population of ecotropic cells producing a high titer, obviating the lengthy selection and characterization of several individual clones for each virus. Infection of ES cells was done by co-culturing the ES cells with a pool of the 6 cell lines. Co-culture infection was carried out for different lengths of time and also with different initial numbers of ES cells. In this way we determined the optimal procedure for infecting ES cells with approximately 10 copies of each retrovirus in the shortest time period. We are now preparing to use these cells to create ES cell injection chimeras and transgenic mice. To screen the new transgenic strains for mutations which disrupt development we will establish standard 3 generation backcross breeding programs. Visible recessive mutations will be scored and correlation between a particular retroviral insertion and the mutation will be determined. For recessive prenatal lethal mutations, we will determine which retroviral insertions are not found in the homozygous state in live born progeny of the backcross populstion. We will also cross the transgenics with strains carrying phenotypically identified but as yet uncloned recessive mutations. The multiplex approach should significantly increase the overall insertional mutagenesis efficiency by maximizing the number of viral insertions that can be analyzed per animal and minimizing the total number of transgenic animals requiring screening. Using several different multiple retroviral insertion strains, we hope to create insertional mutations in many previously unknown genes as well as genes already characterized, and allow these to be cloned for molecular analysis.

Publications:

None

Annual Report

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY
FY 1992

EXPERIMENTAL ONCOLOGY SECTION (Dr. Jeffrey Schlom, Chief)

Monoclonal antibodies define carcinoma-associated and differentiation antigens. These studies involve the generation, characterization, and utilization of monoclonal antibodies (MAbs) directed against antigens associated with human carcinomas. These MAbs are being used to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in several areas of the management of human carcinoma. These include *in vitro* diagnosis via serum assays and/or immunohistopathology, *in vivo* diagnosis such as gamma scanning, and potentially therapy. The MAbs generated can be classified into two groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma versus normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAbs B72.3 and CC49, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; or (b) tissue-associated antigens, such as the colon-associated antigen detected by MAb D612.

Since MAb B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving over 1000 patients, studies were conducted to characterize a series of "second generation" MAbs to the TAG-72 antigen. These studies demonstrated that some of these second generation CC MAbs, such as CC83 and CC49, have a higher affinity constant for TAG-72 than B72.3, and may be better suited than B72.3 for some clinical applications.

The presence of TAG-72 in serum samples from 260 patients with colorectal disease (malignant or benign) has been evaluated using the CA72-4 assay. Approximately 40% of patients with colorectal cancer exhibit elevated levels of this marker, moreover, the presence of positive levels of TAG-72 significantly correlates with advanced stages of disease. These studies suggest that the simultaneous use of TAG-72 and CEA serum markers may be useful in the diagnosis of recurrent disease and may therefore play a role in the clinical management of cancer patients.

Augmentation of tumor antigen expression. The ability to specifically enhance the level of expression of human tumor antigens may be an important advantage in overcoming the limitations associated with antigenic heterogeneity in immunodiagnostic and/or immunotherapeutic clinical protocols. We have reported that human interferons (IFNs), particularly interferon-γ (IFN-γ), can enhance the level of expression of TAG-72 and CEA, two distinct human tumor antigens, on the surface of human carcinoma cells. In particular, experimental studies clearly showed that the increase in CEA expression by a variety of human colon carcinoma cells was accompanied by higher levels of CEA-related mRNA transcripts following IFN-γ treatment. Those studies provided the initial insights into the mechanism(s) involved in CEA regulation by that biological response modifier. Some human colon carcinoma cells were unresponsive to the ability of IFN to augment the level of tumor as well as normal (HLA) antigen expression. IFN treatment of those cells, however, successfully increased 2', 5'A synthetase indicating the presence of a viable IFN receptor. CEA expression was enhanced in those same cells treated with a chloride-substituted analogue of cyclic AMP, 8-Cl-cAMP. Those results indicate the presence of a cAMP-dependent protein kinase pathway through which 8-Cl-cAMP can increase CEA expression. In another study, a potentially novel tumor antigen, immunologically related to CEA and strongly upregulated by IFN-γ, was identified in 6 of 8

human gastric carcinoma cells. Molecular cloning of this 110,000 M_r antigen will address its relationship with that of the large CEA gene family as well as the possible role which the IFNs play in its regulation. Serum samples were collected from a variety of clinical trials that investigated the therapeutic efficacy of different IFNs on a variety of human carcinomas. Analysis of the samples taken before, during and after IFN treatment revealed an increase serum TAG-72 and CEA levels in ~65% of the patients. This occurred in patients whose sera were TAG-72 and/or CEA-positive before IFN therapy, and was not, by and large, observed in sera from patients diagnosed with noncarcinoma malignancies. The findings indicate the ability of therapeutic doses of human IFNs to initiate biological effects at the tumor cell resulting in, in this case, an increase in the amount of human tumor antigen secreted into the blood. Clinical trials are being planned to determine whether one could exploit this action in a diagnostic setting.

Localization and therapy using monoclonal antibodies: Model systems. CC49 is a "second generation" MAb to B72.3, which reacts with the pancarcinoma antigen TAG-72. CC49 has been shown to efficiently target human colon carcinoma xenografts and is currently being evaluated in both diagnostic and therapeutic clinical trials. We have described the construction and characterization of a recombinant single-chain Fv (sFv) of CC49. The sFv was shown to be a M_r 27,000 homogeneous entity which could be efficiently radiolabeled with ^{125}I or ^{131}I. Metabolism studies in mice, using radiolabeled CC49 IgG, $F(ab')_2$, Fab', and sFv, demonstrated an extremely rapid plasma and whole body clearance for the sFv. CC49 sFv plasma pharmacokinetic studies in rhesus monkeys also showed a very rapid plasma clearance. Tumor targeting studies with all four radiolabeled Ig CC49 forms, using the LS-174T human colon carcinoma xenograft model, revealed a much lower percentage injected dose/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 (radiolocalization indices) for the sFv were comparable to or greater than those of the other Ig forms. The CC49 sFv may thus have utility in diagnostic and therapeutic applications for a range of human carcinomas.

^{177}Lutetium (^{177}Lu) is a member of the family of elements known as lanthanides or rare earths. We have demonstrated the first use of a ^{177}Lu-labeled immunoconjugate, ^{177}Lu-CC49, in an experimental therapy model for human carcinoma. ^{177}Lu-CC49 was shown to delay the growth of established LS-174T human colon carcinomas in athymic mice at a single dose of 50 μCi. A single administration of 200 or 350 μCi of ^{177}Lu-CC49 was shown to eliminate established tumors through the 77-day observation period after MAb administration. Dose fractionation experiments revealed that at least 750 μCi of ^{177}Lu-CC49 (250 μCi/week for 3 consecutive weeks) was well tolerated and this dose schedule was able to eliminate the growth of relatively large human colon tumor xenografts in 90% of the animals treated. The merits of the use of ^{177}Lu-labeled immunoconjugated (in particular, ^{177}Lu-CC49) should now be considered in terms of potential novel therapeutics for human carcinoma.

Antibody directed cellular immunotherapy of human carcinoma. We investigated the ability of human recombinant (hr) hrIL-6 to augment antibody directed cell mediated cytotoxicity (ADCC) via MAb D612 using colorectal carcinoma target LS-174T, WiDr and HT-29 cells. A significant increase in ADCC activity was observed after human PMNC were incubated in 100-400 U/ml of hrIL-6. hrIL-6 did not augment non specific (non MAb mediated) cytotoxicity. Enhancement of ADCC activity was blocked by the addition of an antibody against hrIL-6 but not by an antibody to the IL-2 receptor, suggesting the hrIL-6 augmentation of ADCC activity may not be mediated through IL-2. The surface antigen expression and susceptibility to lysis of human IL-6 gene transfected colorectal carcinoma cell line, HT-29 were investigated by flow cytometry and by 24 h ^{111}In release ADCC assay. We found that IL-6 transfected HT-29 cell can secrete a high level of biologically active IL-6. The expression of cell surface antigens in

the transfected HT-29 cells were analyzed. Significant enhancement in the percent of CEA expressing cells as detected by COL-1 MAb but not in the percent of cells expressing HLA-class I, HLA class II, and ICAM-1 antigens as compared to the parental HT-29 cell was observed. The susceptibility to lysis of the transfected HT-29 cells increased significantly in ADCC assay using COL-1 MAb. The increase in ADCC activity correlated well with the increased expression of CEA. These results provide a rationale for use of IL-6 gene transfer into human cells as a possible modality for cancer therapy. We also investigated the tumoricidal properties of D612 MAb alone and in combination with IL-2 activated tumor lymphocytes in athymic mice bearing LS-174T colon tumor xenografts. The resutls demonstrate that the tumoricidal properties of LAK cells and the D612 MAb can be augmented when used together in immunotherapy of human colon cancer xenograft.

Cloning of anti-tumor antigen immunoglobulin genes and modified constructs. The main objective of this research project is to genetically engineer immunoglobulin genes to study structure-function relationships and to generate potentially useful immunological reagents for diagnosis and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) with selective reactivity to various tumor types. These MAbs include those that recognize carcinoembryonic antigen, and a high molecular weight, mucin-like pancarcinoma antigen, TAG-72. The MAbs are currently being evaluated in a number of diagnostic and therapeutic trials on breast, colon and ovarian cancers. The murine MAbs, B72.3 and its second generation counterpart MAb CC49, which recognize TAG-72, have shown promise for being developed into diagnostic and therapeutic agents. A major impediment to the clinical application of murine MAbs is their potential to elicit human anti-mouse antibody (HAMA) response in some patients. This problem can be effectively minimized by genetically replacing the constant region of the mouse antibody with the constant region of human antibody as well as with other genetically engineered manipulations.

Anti-carcinoma monoclonal antibody clinical trials. This project involves the use of monoclonal antibodies (MAbs) in both diagnostic and therapeutic clinical trials. To date, over 1,000 patients have been administered radiolabeled B72.3 in tumor-targeting studies carried out in numerous institutions, with similar findings of approximately 70-80% tumor targeting observed. The selective localization of ^{131}I MAb B72.3 IgG was demonstrated in biodistribution studies in colorectal cancer patients in which the percentage of injected dose of MAb per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% had an RI of at least 3 (*i.e.*, 3 times greater uptake per gram than normal tissues). We have also conducted studies to determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analysis of biopsy specimens).

A phase I therapy trial involving intraperitonel administration of ^{131}I-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity has been conducted. Extremely good localization of tumor lesions in the abdomen was detected. The use of a recombinant/chimeric MAbs has been conducted. Clinical trials involving the use of second and third generation MAb-isotope conjugate are in progress.

Isolation and characterization of genes coding for carcinoma-associated antigens. Monoclonal antibodies (MAbs) have defined several antigens associated with human carcinomas. Two of the most widely studied antigens are carcinoembryonic antigen (CEA) and Tumor-associated glycoprotein-72 (TAG-72). CEA is a 180 kD glycoprotein and TAG-72 is a high molecular weight mucin. Recent studies have demonstrated that CEA is a member of the immunoglobulin supergene family. These include CEA, normal cross-reacting antigen (NCA), biliary glycoprotein (BGP), and human pregnancy-specific beta 1-glycoprotein (SP1).

The use of anti-CEA MAbs for diagnosis and therapy have been explored by a number of labs. In order to assess the ability of CEA to serve as a target for active immunotherapy, a mouse model has been generated in our lab. Mouse tumor cell lines expressing high levels of CEA have been derived, and the characteristics of the expresssed gene products have been analyzed. These tumor cells have been shown to grow in immunocompetent mice, and in preliminary experiments have shown to serve as targets for active immunotherapy, carried out with a CEA-vaccinia construct (see project # Z01 CB 09028-02).

Experiments in other systems have demonstrated the ability of purified proteins produced in a baculovirus system, to boost responses to recombinant proteins produced in vaccinia. Recently, experiments have begun to explore the use of the baculovirus system for production of recombinant CEA.

Active immunotherapy to human carcinoma associated antigens. Active specific immunotherapy is a new and potentially non toxic approach for cancer therapy. Tumor associated antigens could serve as targets for this type of therapy. Carcinoembryonic antigen (CEA) is a 180,000 dalton oncofetal glycoprotein expressed on most gastrointestinal carcinomas and several other human carcinoma types. CEA is generally considered to be weakly immunogenic in humans; that is, little evidence exists for humoral or cell mediated immunity to CEA in normal or cancer patients. The copresentation of CEA with a strong immunogen would represent a logical approach to inducing anti-CEA responses for tumor immunotherapy. Recent advances in recombinant vaccinia virus technology has provided a powerful method for antigenic copresentation. To this end, a 2.4 kb cDNA clone, containing the complete coding sequence of CEA, was isolated from a human colon tumor cell library and was inserted into a vaccinia virus genome. This recombinant construct was characterized by Southern blotting, restriction endonuclease digestion, polymerase chain reaction analysis, and subsequent DNA hybridization. The CEA gene was stably integrated in the vaccinia virus thymidine kinase gene. This recombinant was efficiently replicated upon serial passages in cell culture and in animals. The recombinant virus expressed on the surface of infected cells, a protein product recognized by the MAb COL-1 directed against CEA. Immunization of mice with the recombinant vaccinia virus resulted in a humoral immune response against CEA. Pilot studies demonstrated that the administration of the recombinant vaccinia virus was able to greatly reduce the growth of tumors in mice. This murine colon adenocarcinoma had been transduced with the human CEA gene, expressed human CEA, and grew in syngenic animals. The use of this new recombinant CEA vaccinia virus may provide a new approach in the specific active immunotherapy of human gastrointestinal cancer and other CEA expressing carcinoma types.

CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (H. Cooper, Chief)

We have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous observations have led us to hypothesize that: (a) TM suppression is a causal event in neoplastic transformation; and (b) the oncogenic pathways initiated by a number of different oncogenes and other modalities converge on and act through TM suppression. We have obtained evidence supporting this hypothesis by restoring expression of TM1, one of two suppressed tropomyosins, in the v-Ki-*ras*-transformed NIH3T3 cell line, DT, through the use of a retroviral vector (pBNC) carrying a full length cDNA encoding human TM1. Cell clones expressing the cDNA had elevated levels of TM1 and lost the ability to grow under anchorage-independent consitions. They also did not participate in formation of tumors in athymic mice.

These studies are now being extended. The ability of elevated levels of TM in the transduced cloned to be utilized in the cytoskeleton is under study, as is their participation in the formation

of the physiological dimers in which TM occurs *in vivo*. These studies suggest that complete reversal of the transformed phenotype may require simultaneous restoration of both TM1 and TM2 to permit formation of heterodimeric TM molecules, which are the preferred form *in vivo*. Materials for the insertion of TM2-encoding cDNA into clones already restored in TM1 expression are now being prepared.

TM1 expression has also been restored in the human breast cancer cell line, MCF-7, which we previously showed to be defective in synthesis of this TM isoform. Testing for effects of this restoration on the neoplastic phenotype is currently in progress.

While screening for human epithelial cell tropomyosins in a normal mammary epithelial cell cDNA library, we obtained a full length clone encoding a previously unknown protein which was unrelated to TM. This protein, HME1, is expressed only in epithelial cells, and its expression is reduced in human breast cancer cell lines. It may therefore be a useful marker of epithelial cell differentiation, and also may be helpful in detecting neoplastic change.

TUMOR GROWTH FACTOR SECTION (Dr. David Salomon, Chief)

This section is engaged in the development and utilization of monoclonal antibodies directed against tumor associated antigens for diagnostic and therapeutic purposes. This section has developed a number of MAbs that are in clinical trials. The evaluation of these reagents for their efficacy as targeting agents may enable the early diagnosis of malignancy as well as the metastatic spread of the malignancy. This may lead to the use of these reagents as early serum markers of diagnostic or therapeutic reagents.

Transforming growth factor α (TGFα), amphiregulin (AR) and cripto are proteins that are structurally and in some cases functionally related to epidermal growth factor (EGF) in that TGFα has been 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 TGFα and interference with its biological activity have not been thoroughly examined; and relative levels of expression and biological function of AR and cripto in these malignancies are unknown. Present studies demonstrate that transformation of human mammary epithelial cells with a point-mutated c-Ha-*ras* protooncogene, but not with a c-*erb*B-2 oncogene, results in an increase in TGFα expression. Also, overexpression of human TGFα cDNA in these cells leads to *in vitro* transformation. Addition of an anti-EGF receptor blocking antibody to an anti-TGFα neutralizing antibody can partially or completely inhibit the growth of the ras or TGFα transformed mammary cell, suggesting the establishment of an external autocrine loop. Estrogens can increase TGFα mRNA and protein expression in estrogen-responsive human breast cancer cell lines like MCF-7 cells. Transient transfection assays in MCF-7 cells using a plasmid containing the TGFα promoter ligated to either the chloramphenicol acetyltransferase (CAT) or luciferase genes demonstrate that physiological concentrations of estrogens can induce a 10- to 100-fold increase in the activity of these reporter genes. This suggests that the TGFα promoter contains a *cis*-acting estrogen-responsive element (ERE). MCF-7 cells were infected with a recombinant amphotropic TGFα antisense expression vector. Expression of this antisense RNA leads to partial reduction in basal and estrogen-induced TGFα protein production and to an equivalent degree of inhibition of basal and estrogen-induced proliferation. Specific mRNA transcripts for AR and cripto were detected in ~70% of primary and metastatic colorectal tumors, but only 5% of normal colon or liver tissue expressed these genes. In contrast, cripto mRNA was not expressed in either normal or malignant human mammary tissue whereas AR mRNA was found in ~50% of these samples.

Mammary tumorigenesis in inbred and feral mice. The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with the mouse mammary tumor virus (MMTV). MMTV appears to induce tumors by acting as an insertional mutagen that leads to the activation of expression of a previously silent cellular gene or the rearrangement of a normally expressed gene (*int* genes). We have found a dichotomy in the frequency with which the *wnt*-1 gene is activated in tumors arising within preneoplastic hyperplastic outgrowth lines (6%) and those arising *in situ* (52%) in the mammary glands of C3H breeders. It would appear that *wnt*-1 activation provides a proliferative advantage to transformed mammary epithelial cells in intact C3H mammary glands. We have determined the nucleotide sequence of the 2.3kb RNA species whose expression is activated by MMTV insertion in the *int*-3 locus in mammary tumors. It encodes a 57kD protein which is 50% identical to the intracellular portion of the neurogenic *Drosophila* notch gene product. A common characteristic of these proteins is six nearly contiguous 32 amino acid repeats which are bounded by the PEST amino acid sequence motif that is characteristic of proteins having a rapid turnover. We have used the "normal" HC11 mouse mammary epithelial cell line to study the biological activity of the *int*-2 and *int*-3 gene products. *Int*-2 is a member of the fibroblast growth factor (FGF) gene family. Activation of expression of either *int*-2 or *int*-3 in HC11 cells induces anchorage-independent growth in soft agar. Moreover, the autocrine expression of *int*-2 in HC11 cells abrogates their requirement for either epidermal growth factor or bFGF priming prior to induction of beta-caesin expression with lactogenic hormones. A transgenic mouse strain has been established containing activated *int*-3 as the transgene. Focal and often multiple poorly differentiated mammary and salivary gland adenocarcinomas occur in 100% of the transgenic mice between 2 and 7 months of age. Significantly, mammary glands were arrested in development and were lactation deficient in all female *int*-3 mice. In other studies we have developed a novel approach to introducing genes into primary mammary epithelial cells to test their biological activity in mammary fat pads, using a retroviral shuttle vector containing a LacZ reporter gene.

The identification and characterization of human genes associated with neoplasia. The etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken an ongoing program that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association with the patients history, characteristics of the tumor, and the patients prognosis. The most frequent type of mutation is loss of heterozygosity (LOH) at specfic regions of the cellular genome in tumor DNA. In previous studies we have found LOH on chromosomes 1p, 1q, 3p, 11p, 13q, 17p, 17q, and 18q. Our current results demonstrate LOH of the c-met proto-oncogene on chromosome 7q in 40.5% of the tumor DNAs. This genetic alteration was not associated with the lymph node status of the patient nor other standard prognostic features of the tumor. However, patients having tumors with LOH on chromosome 7q had a significantly shorter disease-free interval ($p=0.00022$) and overall survival ($p=0.0036$). A separate panel of 96 primary breast tumors was evaluated for their proliferative index by their ability to incorporate BrdU in culture. A significant association ($p=0.022$) was found between those tumors having an elevated BrdU labeling index and LOH at the pYNZ22.1 locus on chromosome 17p. In contrast, no association was found between the tumor BrdU labeling index and LOH at the more telemeric locus p144D6 on chromosome 17p nor LOH on chromosomes 1p, 3p, 13q, or 18q. To determine whether the p53 tumor suppressor gene is a target for LOH on chromosome 17p we examined 26 tumors by RNAse protection assays and nucleotide sequence analysis for p53 mutations. The same three tumors

were found to have point mutations in the p53 gene. We also did single strand conformation polymorphism (SSCP) analysis which proved more sensitive in the detection of mutaions. Taken together the results showed that a total of 12 p53 mutations in 11 tumors (46%). Currently we are determining whether p53 mutations are linked to the proliferative index of the tumor.

CELLULAR BICHEMISTRY SECTION (Y.S. Cho-Chung, Chief)

The Cellular Biochemistry Section studies the control mechanism of cell growth and differentiation by cyclic AMP (cAMP). Use of new derivatives of cAMP, site-selective cAMP analogs, whose effects far exceed that of parent cAMP or the previously studied cAMP analogs, antisense strategy, and retroviral vector-mediated gene transfer, we anticipate achieving our research goals in the following areas: (1) a better understanding of the regulatory mechanisms of cell growth and differentiation, (2) a clearer definition of the derangement of cAMP-effector function in neoplastic transformation and progression, and (3) improved management of human cancers by providing the non-toxic biological agents as antineoplastics and chemopreventives. Our studies being conducted in two projects are summarized below.

Our hypothesis is that cAMP-dependent protein kinases are crucial effectors in tumorigenesis. cAMP acts by binding to the regulatory subunits of cAMP-dependent protein kinase. Two such subunits exist, RI and RII, which interact with a common catalytic subunit and are present in normal cells as a specific physiological ratio; departure from the normal balance of these two isoforms of the subunits may lead to the induction of malignant transformation. cAMP binds to RI and RII; however, these cAMP receptor proteins transduce opposite signals, the RI being stimulatory and the RII inhibitory of cell proliferation. This conclusion was drawn from the studies that employed independent experimental approaches: the use of site-selective cAMP analogs that, unlike parent cAMP, are able to differentiate between the binding sites on RI and RII; antisense oligonucleotides, those that are able to selectively inhibit the function of RI and RII; and transfer and overexpression of RI or RII gene by a retroviral vector.

These studies demonstrated that restoration of the normal balance between RI and RII is of great potential in cancer therapy. Thus, these studies contribute to understanding the mechanism of cAMP control of cell growth and differentiation and provide new approaches to the treatment of cancer.

OFFICE OF THE CHIEF (Dr. Jeffrey Schlom, Chief)

"Anti-oncogenes": The analysis of cellular resistance to transformation. We have constructed a cDNA library from a *ras* revertant cell line, in a eukaryotic expression vector and screened this library for cDNAs which are 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. Two cDNAs isolated using this strategy have been found on secondary screening to be capable of suppressing the *ras* transformed phenotype. The first of these cDNAs, referred to as *rsp*-1, is a novel gene which specifically suppresses v-Ki-*ras* and v-Ha-*ras* transformation of fibroblasts and epithelial cells. The *rsp*-1 protein contains a series of leucine based repeats homologous to those found in the putative ras binding region of yeast adenylyl cyclase. These findings in conjunction with the fact that it is a phylogenetically highly conserved protein suggests that rsp-1 may physically associate with *ras* p21. In addition, we have identified a small RNA, 4.5S RNA, as 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. Our current efforts are

aimed at determining the mechanisms by which these two molecules disrupt v-*ras* signal transduction.

Hormones and growth factors in development of mammary glands and tumorigenesis. 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 milleu of the target cell. Our emphasis has been on the interactions of prolactin (Prl), thyroid hormone, and estrogens with recent work also examining how epidermal growth factor (EGF), and EGF-like growth factors are affected by the interplay of these three classical hormones. In addition, we have explored the relationship of a membrane associated antilactogen binding site (ALBS) to the lactogenic hormone receptor on human breast cancer cell growth in culture. 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 α-TGF can promote full lobulo-alveolar development *in vitro*. This effect is not inhibited by β-TGF. The primed mammary gland is more sensitive to α-TGF than to EGF. Prl induced growth of the mouse mammary epithelial cell NOG-8 appears to involve 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. 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 may be intimately associated with the Prl receptor. The antiprolactin action of tamoxifen, working through the ALBS, may have important clinical implications.

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

PROJECT NUMBER
Z01 CB 05190-12 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens

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

Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
Patricia Horan Hand	Chemist	LTIB, DCBDC, NCI
Diane Eggensperger	Senior Staff Fellow	LTIB, DCBDC, NCI
Sam Zaremba	Special Volunteer	LTIB, DCBDC, NCI
Jack Greiner	Chemist	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

Fiorella Guadagni, Regina Elena Cancer Institute, Rome, Italy
Patrizia Ferroni, Department of Experimental Medicine, Rome, Italy

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.3	1.7	2.6

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☐ (a1) Minors
☐ (a2) Interviews

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

These studies involve the generation, characterization, and utilization of monoclonal antibodies (MAbs) directed against antigens associated with human carcinomas. These MAbs are being used to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in several areas of the management of human carcinoma. These include *in vitro* diagnosis via serum assays and/or immunohistopathology, *in vivo* diagnosis such as gamma scanning, and potentially therapy. The MAbs generated can be classified into two groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma versus normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAbs B72.3 and CC49, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; or (b) tissue-associated antigens, such as the colon-associated antigen detected by MAb D612.

Since MAb B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving over 1000 patients, studies were conducted to characterize a series of "second generation" MAbs to the TAG-72 antigen. These studies demonstrated that some of these second generation CC MAbs, such as CC83 and CC49, have a higher affinity constant for TAG-72 than B72.3, and may be better suited than B72.3 for some clinical applications.

The presence of TAG-72 in serum samples from 260 patients with colorectal disease (malignant or benign) has been evaluated using the CA72-4 assay. Approximately 40% of patients with colorectal cancer exhibit elevated levels of this marker, moreover, the presence of positive levels of TAG-72 significantly correlates with advanced stages of disease. These studies suggest that the simultaneous use of TAG-72 and CEA serum markers may be useful in the diagnosis of recurrent disease and may therefore play a role in the clinical management of cancer patients.

Major Findings

Monoclonal antibody (MAb) B72.3 has been shown to be of potential utility in the management of human carcinoma via its use in (a) the targeting of carcinoma lesions in colorectal and ovarian cancer patients, (b) immunohistochemical analyses of biopsies and effusions, and (c) serum assays to help define the presence of carcinoma. The B72.3-reactive antigen, designated tumor-associated glycoprotein 72 (TAG-72), has been characterized as a high molecular weight glycoprotein with the properties of a mucin. TAG-72, has been purified from a human colon carcinoma cancer xenograft and used as an immunogen to generate second generation MAbs. Twenty-eight of these MAbs, designated CC (colon cancer), were shown to be reactive with tumor-associated glycoprotein 72; direct-binding radioimmunoassays, Western blotting, live cell surface binding assays, liquid competition radioimmunoassays, and affinity constant measurements distinguished CC MAbs from each other and from B72.3. A novel tumor marker, tumor-associated glycoprotein-72 (TAG-72), has been identified using MAb B72.3. Using immunohistochemical techniques, TAG-72 has been found in carcinomas of various origin including colon, stomach, breast, lung, prostate, and ovary, as well as in body fluids. The presence of TAG-72 in serum samples from 260 patients with colorectal disease (malignant or benign) has been evaluated using the CA72-4 assay. Approximately 40% of patients with colorectal cancer exhibit elevated levels of this marker; moreover, the presence of positive levels of TAG-72 significantly correlates with advanced stages of disease, suggesting that TAG-72 may be a good marker of advanced colorectal cancer. Only 2% of the patients diagnosed with colorectal disease has elevated TAG-72 serum levels indicating the high specificity of this marker. A comparative study with carcinoembryonic antigen (CEA) serum levels showed a complementarity of the two tumor markers; in fact, 49.6% of CEA negative cases scored positive for TAG-72. A longitudinal evaluation of TAG-72 serum levels in 31 patients with malignant disease was performed. The results indicate that patients with increasing TAG-72 serum levels postoperatively may be indicative of recurrent disease. In 60% of patients in which significant changes of CEA levels could not be detected, TAG-72 showed rising positive levels prior to clinical evidence of recurrent disease. These results suggest that the simultaneous use of TAG-72 and CEA serum markers may be useful in the diagnosis of recurrent disease and therefore play and important role in the clinical management of cancer patients. In another study, 82 patients diagnosed with gastrointestinal (GI) adenocarcinoma were evaluated before and for 26 months after primary tumor resection for the presence of two serum tumor markers, TAG-72 and CEA. Elevated TAG-72 and CEA serum levels were found preoperatively in 32 (39%) and 34 (41%) of the 82 patients, respectively. The percentage of patients with elevated serum levels of either TAG-72 or CEA was 56.1% (46 of 82). Twelve (15%) patients who had normal CEA serum levels had elevated TAG-72 serum levels, and conversely, serum from 14 (17%) patients who were TAG-72 negative were CEA positive. Forty-five of the 82 patients were diagnosed with advanced disease (i.e., Stages C and D for colorectal, Stages III and IV for stomach), and 29 (64.4%) and 26 (57.8%) of those patients had elevated serum levels of TAG-72 or CEA, respectively. Elevated levels of either TAG-72 or CEA, however, were found in sera of 82.2% of patients with advanced GI cancer, which is an increase of 24.4% over the use of CEA antigen alone as a marker of disease. The measurement of both TAG-72 and CEA may improve the diagnosis of patients with GI malignant disease due to the apparent complementary association which exists between these tumor markers. Serum TAG-72 and CEA levels were monitored in 31 patients for varying lengths of time after resection of the carcinoma; 11 patients developed recurrent disease. Sera from nine of 11 (81.8%) of these patients had elevated TAG-72 levels and six of 11 (54%) had elevated CEA levels. Tumor marker elevations were observed either before (35 to 166 days) or at the time of diagnosis of recurrence. The elevation of one or both markers correlated with the clinical status in ten of 11 (90.9%) 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 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.

We have utilized MAb B72.3 and the 18 second generation MAbs (generated using purified TAG-72 obtained from a colon carcinoma xenograft as immunogen) to construct a serological map of the TAG-72 molecule. Immunodepletion analyses utilizing 11 of the anti-TAG-72 MAbs indicated that each recognizes the same molecule or population of molecules. Nineteen competition radioimmunoassays were developed and 19 purified competitor immunoglobulins were used in each assay. The patterns of cross-competition indicated the presence of a complex array of tumor-associated epitopes on the TAG-72 molecule. Some of the MAbs recognized epitopes that were structurally or spatially related to one another, but none appeared to recognize identical epitopes. The spectrum of inhibitory reactivities of these MAbs for TAG-72 binding varied from extremely restricted to more broad inhibition. These serological mapping studies thus provide information as to the range and nature of the epitopes expressed on the TAG-72 molecule, help form the basis for selecting alternative anti-TAG-72 MAbs for use in potential clinical applications, and further define the nature of this oncofetal antigen.

MAb D612 recognizes an antigen expressed on the cell surface of normal and malignant gastrointestinal epithelium. It is a murine IgG2a/k which has been previously shown to mediate killing of human colon carcinoma cells using human effector cells (which could be enhanced in the presence of interleukin-2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of MAb D612 immunoprecipitates of extracts of L-[^{35}S]methionine-, L-[H]leucine-, and D-[^{3}H]glucosamine-labeled human colon carcinoma cells showed that the D612 antigen is a Mr 48,000 glycoprotein. Similar estimates of molecular mass were obtained from SDS-PAGE analyses of MAb D612 immunoprecipitates of radioiodinated extracts of surgically resected colon carcinoma and adjacent normal colonic mucosa. D612 antigen was not detectable in immunoprecipitates of supernatant media from radiolabeled cell cultures, suggesting that the antigen is not readily shed from the surface of cultured cells. The D612 antigen was shown to be clearly distinct from previously described gastrointestinal carcinoma-associated glycoproteins: the D612 antigen shows a migration pattern on SDS-PAGE distinct from those of the antigens recognized by MAbs KS1/4 and GA733, and reciprocal immunodepletion analyses of D-[^{3}H]glucosamine-labeled colon carcinoma cells utilizing MAbs D612 and GA733 revealed no cross-reactivity between these antibodies.

Flow cytometric analysis of phosphatidylinositol-specific phospholipase C-treated colon carcinoma cells revealed no loss of D612 antigen from the cell surface, suggesting that the mechanism of attachment of the D612 antigen to the cell surface does not involve linkage to a phosphatiylinositol glycan. Radioiodination of the D612 antigen in a plasma membrane-enriched cell fraction by the photoactivatable carbene-generating reagent, 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazarine, suggests that the D612 antigen polypeptide penetrates the lipid bilayer of the plasma membrane. It has been determined by Scatchard analysis that the number of binding sites for MAb D612 on the LS-174T human colorectal carcinoma cell line is 4.8×10^{5}. MAb D612 was found to have a Ka of approximately $1.3 \times 10^{9} M^{-1}$.

A novel gene product which is immunologically related to carcinoembryonic antigen (CEA) and constitutively expressed by six of eight human gastric carcinoma cell lines has been described. The antigen was initially identified by the differential binding patterns of four MAbs which recognize the putative M_r 180,000 CEA and/or the M_r 90,000 CEA-related gene product, NCA (normal cross-reacting antigen). Western blot analyses using CEA- and NCA-specific complementary DNA

probes did not identify any specific CEA or NCA transcripts in polyadenylate-selected mRNA isolated from the Hs746T cells, Likewise, a probe designed to hybridize with different CEA-related family members failed to identify a CEA-related message in the Hs746T cells. Phosphatidylinositol phospholipase C treatment failed to release the M_r 110,000 antigen from the surface of the Hs 746T cells, suggesting that membrane attachment of this novel antigen is not via a glycosyl-phosphatidylinositol anchor. Finally, primers that amplify the 420 base pairs of the immunoglobulin-like domain of CEA and NCA detected an appropriately sized product in GaCa cells using the polymerase chain reaction method. Thus, a potentially novel gene product coding for an M_r 110,000 antigen has been identified in human gastric carcinoma cells. Immunologically, the antigen shares reactive epitopes with CEA and its related NCA gene product; however, Northern blot analyses, polymerase chain reaction, and phosphatidylinositol phospholipase C results suggest that the antigen may be, at best, a distant relative of the CEA gene family.

Publications

Bast RC, Knauf S, Epenetos A, Dhokia B, Daly L, Tanner M, Soper J, Creasman W, Gall S, Knapp RC, Zurawski VR, Schlom J, Kufe DW, Ritts RE. Coordinate elevation of serum markers in ovarian cancer but not in benign disease, Cancer 1991;68:1758-63.

Fernsten PD, Primus FJ, Greiner JW, Simpson JF, Schlom J. Characterization of the colorectal carcinoma-associtaed antigen defined by monoclonal antibody D612, Cancer Res 1991;51:926-34.

Guadagni F, Roselli M, Amato T, Cosimelli M, Mannella E, Perri P, Abbolito MR, Cavaliere R, Colcher D, Greiner JW, Schlom J. Tumor-associated glycoprotein-72 (TAG-72) serum levels complement carcinoembryonic antigen (CEA) levels in monitoring patients with gastrointestinal carcinoma: a longitudinal study, Cancer 1991;68:2443-50.

Guadagni F, Roselli M, Ferroni P, Amato T, Colcher D, Greiner JW, Schlom J. Clinical evaluation of the new tumor marker TAG-72, Anticancer Res 1991;11:1389-94.

Guadagni F, Roselli M, Amato T, Cosimelli M, Mannella E, Tedesco M, Grassi A, Casale V, Cavaliere R, Greiner JW, Schlom J. Clinical evaluation of serum tumor-associated glycoprotein-72 as a novel tumor marker for colorectal cancer patients, J Surg Oncol 1991;2:16-20.

Simpson JF, Primus FJ, Schlom J. Complementation of expression of carcinoembryonic antigen and tumor associated glycoprotein-72 (TAG-72) in human colon adenocarcinomas, Int J Biol Markers 1991;6:83-90.

Shimada S, Ogawa M, Schlom J, Greiner JW. Identification of a novel tumor-associated 110 kD gene product in human gastric carcinoma cells that is immunologically related to CEA, Cancer Res 1991;51:5694-703.

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

PROJECT NUMBER
Z01 CB 09008-11 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Localization and Therapy Using Monoclonal Antibodies: Model Systems

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

Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
Patricia Horan Hand	Chemist	LTIB, DCBDC, NCI
Diane Milenic	Microbiologist	LTIB, DCBDC, NCI
Margaret Schott	Expert	LTIB, DCBDC, NCI
Diane Eggensperger	Senior Staff Fellow	LTIB, DCBDC, NCI
Takashi Yokota	Expert	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Otto Gansow, ROB, DCT, NCI; George Hinkle, Ohio State U., Columbus, Ohio; Dave Houchens, Battelle Memorial Institute, Columbus, Ohio; William Goeckeler, Dow Chemical Co., Midland, MI; Marc Whitlow, Enzon; Gaithersburg, Bethesda, MD

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
9.9	4.5	5.4

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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.)

CC49 is a "second generation" MAb to B72.3, which reacts with the pancarcinoma antigen TAG-72. CC49 has been shown to efficiently target human colon carcinoma xenografts and is currently being evaluated in both diagnostic and therapeutic clinical trials. We have described the construction and characterization of a recombinant single-chain Fv (sFv) of CC49. The sFv was shown to be a Mr 27,000 homogeneous entity which could be efficiently radiolabeled with 125-I or 131-I. Metabolism studies in mice, using radiolabeled CC49 IgG, F(ab')2, Fab', and sFv, demonstrated an extremely rapid plasma and whole body clearance for the sFv. CC49 sFv plasma pharmacokinetic studies in rhesus monkeys also showed a very rapid plasma clearance. Tumor targeting studies with all four radiolabeled Ig CC49 forms, using the LS-174T human colon carcinoma xenograft model, revealed a much lower percentage injected dose/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 (radiolocalization indices) for the sFv were comparable to or greater than those of the other Ig forms. The CC49 sFv may thus have utility in diagnostic and therapeutic applications for a range of human carcinomas.

177-Lutetium (177-Lu) is a member of the family of elements known as lanthanides or rare earths. We have demonstrated the first use of a 177-Lu-labeled immunoconjugate, 177-Lu-CC49, in an experimental therapy model for human carcinoma. 177-Lu-CC49 was shown to delay the growth of established LS-174T human colon carcinomas in athymic mice at a single dose of 50 µCi. A single administration of 200 or 350 µCi of 177-Lu-CC49 was shown to eliminate established tumors through the 77-day observation period after MAb administration. Dose fractionation experiments revealed that at least 750 µCi of 177-Lu-CC49 (250 µCi/week for 3 consecutive weeks) was well tolerated and this dose schedule was able to eliminate the growth of relatively large human colon tumor xenografts in 90% of the animals treated. The merits of the use of 177-Lu-labeled immunoconjugated (in particular, 177-Lu-CC49) should now be considered in terms of potential novel therapeutics for human carcinoma.

Major Findings

Monoclonal antibody (MAb) B72.3, a murine IgG, has been developed in this laboratory and shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, including colon, ovarian, breast, lung, pancreatic and gastric malignancies. ^{131}I-B72.3 IgG has been used successfully *in vivo* for the detection of human malignancies including colorectal, ovarian, breast and lung carcinomas after both i.v. and i.p. administration. B72.3 was, therefore, chosen as the antibody to use for the evaluation of different radiolabeling methodologies and MAb modification techniques that may alter the biodistribution of MAbs.

Biodistribution for five different backbone-substituted derivitives of bifunctional chelates SCN-Bz-DTPA (Mx-DTPA, 1M3B-DTPA, 1B3M-DTPA, GEM-DTPA and 2B-DTPA) linked to MAb B72.3 were compared to that of the parent molecule after labeling with ^{111}Indium. Athymic mice, bearing human colon carcinoma xenografts (LS-174T) were injected i.v. to determine the biodistribution of the MAb chelate conjugates. Three of the MAb metal chelate conjugates (Mx-DTPA, 1M3B-DTPA, and 1B3M-DTPA), labeled with ^{111}In showed efficient and stable tumor localization as well as a slower blood clearance rate than SCN-Bz-DTPA, GEM-DTPA or 2B-DTPA MAb chelate conjugates. Major differences were also seen in a normal organ uptake, especially liver and spleen. Tumor-to-liver ratios rose as a function of time for Mx-DTPA, 1M3B-DTPA, and 1B3M-DTPA MAb chelate conjugates with virtually no accumulation of the radiometal into this organ, as revealed by no increase in the liver-to-blood values. Small accretion in normal liver was noted for SCN-Bz-DTPA, GEM-DTPA or 2B-DTPA MAb chelate conjugates. The results demonstrate that the use in vivo of backbone-substituted forms of the SCN-Bz-DTPA, such as Mx-DPTA, 1M3B-DTPA, and 1B3M-DTPA bound to MAbs can reduce uptake of indium to normal organs while maximizing the dose to tumor.

CC49 is a "second generation" monoclonal antibody to B72.3, which reacts with the pancarcinoma antigen TAG-72. CC49 has been shown to efficiently target human colon carcinoma xenografts and is currently being evaluated in both diagnostic and therapeutic clinical trials. We have described the construction and characterization of a recombinant single-chain Fv (sFv) of CC49. The 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 had relative binding affinities 8-fold lower than the dimeric F(ab')$_2$ and intact IgG. Nonetheless, the ^{131}I-labeled sFv was shown to bind biopsies of TAG-72 expressing tumors. Metabolism studies in mice, using radiolabeled CC49 IgG, F(ab')$_2$, Fab', and sFv, demonstrated an extremely rapid plasma and whole body clearance for the sFv. CC49 sFv plasma pharmacokinetic studies in rhesus monkeys also showed a very rapid plasma clearance ($T_{1/2}\alpha$ of 3.9 min and $T_{1/2}\beta$ of 4.2 h). Tumor targeting studies with all four radiolabeled Ig CC49 forms, using the LS-174T human colon carcinoma xenograft model, revealed a much lower percentage injected dose/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 (radiolocalization indices) 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. Gamma scanning studies also showed that ^{131}I-CC49 sFv could efficiently localize tumors. The CC49 sFv may thus have utility in diagnostic and perhaps therapeutic applications for a range of human carcinomas.

^{177}Lutetium (^{177}Lu) is a member of the family of elements known as lanthanides or rare earths. Monoclonal antibody (MAb) CC49, a murine IgG1, which is reactive with the tumor-

associated antigen TAG-72, has been shown previously to react with a wide range of human carcinomas; CC49 reacts to a different epitope on the TAG-72 nlolecule than MAb B72.3 and has a higher binding affinity. We have reported the first use of a ^{177}Lu-labeled immunoconjugate, ^{177}Lu-CC49, in an experimental therapy model for human carcinoma. ^{177}Lu-CC49 was shown to delay the growth of established LS 174T human colon carcinomas in athymic mice at a single dose of 50 μCi. Overt toxicity was observed with the administration of approximately 500 μCi of ^{177}Lu-CC49 in which 5 of 9 mice died of apparent marrow toxicity. A single administration of 200 or 350 μCi of ^{177}Lu-CC49, however, was shown to eliminate established tumors through the 77-day observation period after MAb administration. Dose fractionation experiments revealed that at least 750 μCi of ^{177}Lu-CC49 (250 μCi/week for 3 consecutive weeks) was well tolerated in that 9 of 10 mice survived. Moreover, this dose schedule was able to eliminate the growth of relatively large (300 mm^3) human colon tumor xenografts in 90% of the animals treated. Single-dose and dose fractionation studies were also carried out with an isotype-matched control MAb, ^{177}Lu-MOPC-21. In all dose schedules, a large differential was seen between the therapeutic effects of the ^{177}Lu-CC49 *versus* that of the ^{177}Lu-control MAb. The merits of the use of ^{177}Lu-labeled immunoconjugates (in particular, ^{177}Lu-CC49) should now be considered in terms of potential novel therapeutics for human carcinoma.

Publications

Colcher D, Milenic DE, Ferroni P, Roselli M, Schlom J. In vivo and in vitro clinical applications of monoclonal antibodies against TAG-72, Nucl Med Biol 1991;18:395-401.

Milenic DE, Yokota T, Filupla DR, Finkelman MAJ, Dodd SW Wood JF, Whitlow M, Snoy P, Schlom J. Construction, binding properties, metabolism and tumor targeting of a single chain Fv (sFv) derived from the pan carcinoma MAb CC49, Cancer Res;199151:6363-71.

Roselli M, Schlom J, Gansow OA, Brechbiel MW, Mirzadeh S, Pippin CG, Milenic DE, Colcher D. Comparative biodistribution studies of DTPA-derivative bifunctional chelates for radiometal labeled monoclonal antibodies, Nucl Med Biol 1991;18:389-94.

Schlom J, Milenic DE, Roselli M, Colcher D, Bird R, Johnson S, Hardman KD, Guadagni F, Greiner JW. New concepts in monoclonal antibody based radioimmunodiagnosis and radioimmunotherapy of carcinoma, Nucl Med Biol 1991;18:425-35.

Schlom J, Siler K, Milenic DE, Eggensperger D, Miller LS, Houchens D, Cheng R, Kaplan D, Goeckeler W. Monoclonal antibody based therapy of a human tumor xenograft with ^{177}Lutetium (^{177}Lu)-Labeled immunoconjugate, Cancer Res 1991;51:2889-96.

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

PROJECT NUMBER
Z01 CB 09009-11 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Augmentation of Tumor Antigen Expression

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

Jack Greiner	Chemist	LTIB, DCBDC, NCI
Carol Nieroda	Staff Fellow	LTIB, DCBDC, NCI
Claudio Dansky- Ullmann	Special Volunteer	LTIB, DCBDC, NCI
Shinya Shimada	Visiting Fellow	LTIB, DCBDC, NC
Y.S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. S. Pestka, UMDNJ - Robert Wood Johnson Medical School, Piscataway, NJ; Drs. E. Borden, D. Smalley, and D. Goldstein, U. Wisconsin Medical Center, Madison, WI

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.6	2.6	1.0

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☐ (a2) Interviews

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

The ability to specifically enhance the level of expression of human tumor antigens may be an important advantage in overcoming the limitations associated with antigenic heterogeneity in immunodiagnostic and/or immunotherapeutic clinical protocols. We have reported that human interferons (IFNs), particularly interferon-γ (IFN-γ), can enhance the level of expression of TAG-72 and CEA, two distinct human tumor antigens, on the surface of human carcinoma cells. In particular, experimental studies clearly showed that the increase in CEA expression by a variety of human colon carcinoma cells was accompanied by higher levels of CEA-related mRNA transcripts following IFN-γ treatment. Those studies provided the initial insights into the mechanism(s) involved in CEA regulation by that biological response modifier. Some human colon carcinoma cells were unresponsive to the ability of IFN to augment the level of tumor as well as normal (HLA) antigen expression. IFN treatment of those cells, however, successfully increased 2', 5'A synthetase indicating the presence of a viable IFN receptor. CEA expression was enhanced in those same cells treated with a chloride-substituted analogue of cyclic AMP, 8-Cl-cAMP. Those results indicate the presence of a cAMP-dependent protein kinase pathway through which 8-Cl-cAMP can increase CEA expression. In another study, a potentially novel tumor antigen, immunologically related to CEA and strongly upregulated by IFN-γ, was identified in 6 of 8 human gastric carcinoma cells. Molecular cloning of this 110,000 Mr antigen will address its relationship with that of the large CEA gene family as well as the possible role which the IFNs play in its regulation. Serum samples were collected from a variety of clinical trials that investigated the therapeutic efficacy of different IFNs on a variety of human carcinomas. Analysis of the samples taken before, during and after IFN treatment revealed an increase in serum TAG-72 and CEA levels in ~65% of the patients. This occurred in patients whose sera were TAG-72 and/or CEA-positive before IFN therapy, and was not, by and large, observed in sera from patients diagnosed with noncarcinoma malignancies. The findings indicate the ability of therapeutic doses of human IFNs to initiate biological effects at the tumor cell resulting, in this case, an increase in the amount of human tumor antigen secreted into the blood. Clinical trials are being planned to determine whether one could exploit this action in a diagnostic setting.

Major Findings

It is well documented that recombinant human interferons can regulate the expression of CEA in selected human carcinoma cells. Treatment of several moderately differentiated human colon carcinoma cells with interferon can increase the percentage of cells expressing CEA as well as the amount of CEA expressed per cell. This higher level of CEA expression, or any other human tumor antigen, may be an important advantage in the use of monoclonal antibodies (MAbs) in diagnostic and/or therapeutic setting where antigen heterogeneity is a consideration. Concomitant with the increase in CEA expression as a result of interferon treatment is an increase in CEA-related mRNA transcripts which indicates that transcriptional and/or posttranscriptional events may be involved. CEA expression in some human colon carcinoma cells is unaltered following interferon treatment. Those same cells have viable surface interferon receptors as evidence by the changes induced in other interferon-sensitive gene products (*i.e.*, 2'-5'A synthetase). A study was designed to determine whether an analogue of cyclic AMP, 8-Cl-cAMP, could regulate CEA expression on a highly differentiated human colon carcinoma cell, LS-174T, that previously had been reported to be unresponsive to the human interferons.

Treatment of human colorectal tumor cells (LS-174T, HT29, and WiDr) with analogues of cyclic AMP (cAMP) (dibutyryl-cAMP and 8-Cl-cAMP) selectively enhanced the expression of carcinoembryonic antigen (CEA). Dose and temporal kinetics results revealed that 8-Cl-cAMP was approximately 1OO-fold more potent than dibutyryl-cAMP for increasing CEA expression. Results demonstrated that 8-Cl-cAMP treatment of LS-174T quantitatively increased CEA levels in cell extracts 2-fold, increased anti-CEA monoclonal antibody (MAb) binding to the tumor cell surface, and induced the appearance of CEA-related mRNA transcripts. The findings suggest that 8-Cl-cAMP is capable of regulating CEA expression at transcriptional and/or post-transcriptional levels. Other human tumor cells, as well as normal cell types which do not constitutively express CEA, remained CEA-negative following 8-Cl-cAMP treatment. Moreover, the level of expression of other human tumor antigens as well as antigens of the major histocompatibility complex were not changed by 8-Cl-cAMP treatment, suggesting some selectivity for CEA regulation by this cAMP analogue. *In vivo* administration of 8-Cl-cAMP to athymic mice bearing LS-174T tumor xenografts increased the amount of anti-CEA MAb bound to tumor extracts as well as the tumor localization of a radionuclide conjugated anti-CEA MAb. The results indicate that 8-Cl-cAMP can selectively upregulate CEA expression on human colorectal tumor cells *in vitro* and *in vivo*. Interestingly, IFN-γ treatment of the LS-174T cells fails to enhance or induce expression of CEA or any of the histocompatibility leukocyte antigens. Thus, 8-Cl-cAMP treatment regulates CEA expression through another cellular pathway which may involve cAMP-dependent protein kinase.

The augmentation of tumor antigen expression by the recombinant human interferons is a selective event. The level of expression of such well characterized human tumor antigens as 17-1A and CA19-9 and a more recently described 48 kD antigen, recognized by MAb D612, remains unchanged on the surface of human tumor cells treated with interferon. In addition to CEA, studies have shown that the expression of the high molecular mucin, TAG-72, can be augmented by treating human carcinoma cells with interferon. A potentially novel gene product which is immunologically related to CEA and whose level of expression is strongly upregulated by interferon-gamma (IFN-γ) was found in human gastric carcinoma cells.

The antigen was initially identified by the differential binding patterns of four monoclonal antibodies (MAbs) which recognize the putative M_r 180,000 CEA and/or the M_r 90,000 CEA-related gene product, NCA (normal cross-reacting antigen). Western blot analyses of partially purified membrane fractions prepared from Hs746T gastric carcinoma cells identified an M_r 110,000 antigen. Northern blot analyses using CEA- and NCA-specific complementary DNA probes did not identify any specific CEA or NCA transcripts in polyadenylate-selected mRNA isolated from the Hs746T cells. Likewise, a probe designed to hybridize with different CEA-related family members failed to identify a CEA-related message in the Hs746T cells. Subsequent studies revealed that interferon-γ (IFN-γ) treatment substantially increased the level of expression of the M_r 110,000 antigen on the Hs746T and five other gastric cell types that constitutively expressed the antigen. IFN-γ treatment also *de novo* induced the expression of the M_r 110,000 antigen on the surface of GaCa gastric carcinoma cells. A high percentage of Hs746T (i.e., 85%) and GaCa (approximately 75%) gastric carcinoma cells expressed the M_r 110,000 antigen after IFN-γ treatment; yet, neither cell type expressed CEA or NCA as measured by the binding of the anti-CEA MAb, COL-1, or B6.2, an anti-NCA MAb. In contrast to CEA and NCA, phosphatidylinositol phospholipase C treatment failed to release the M_r 110,000 antigen from the surface of the Hs746T or IFN-γ-treated GaCa cells, suggesting that membrane attachment of this novel antigen is not via a glycosyl-phosphatidylinositol anchor. Finally, primers that amplify the 420 base pairs of the immunoglobulin-like domain of CEA and NCA detected an appropriately sized product in untreated as well as IFN-γ treated GaCa cells using the polymerase chain reaction method. Thus, a potentially novel gene product coding for an M_r 110,000 antigen that is strongly upregulated by IFN-γ has been identified in human gastric carcinoma cells. Immunologically, the antigen shares reactive epitopes with CEA and its related NCA gene product; however, Northern blot analyses, polymerase chain reaction, and phosphatidylinositol phospholipase C results suggest that the antigen may be, at best, a distant relative of the CEA gene family.

The ability to increase the expression of a tumor antigen on the surface of human carcinoma cells may play an important role in improving the targeting of a MAb labeled with a radionuclide, drug or toxin. The enhanced MAb targeting may, therefore, augment the efficacy of the MAb in an immunodiagnostic or immunotherapeutic environment. Another potential role that interferons may play involves the concomitant increase in TAG-72 and CEA secreted from human tumor cells treated with the cytokine. Experimental studies revealed that interferon treatment of human tumor cells which express high surface membrane levels of CEA, increased the release of the antigen into the culture medium. Moreover, the moderately differentiated colon carcinoma cells whose membrane CEA expression was increased by interferon treatment begins to secrete measurable quantities of the antigen. Thus, in those experimental studies, not only can interferon treatment increase the amount of antigen released from the tumor cell surface, but, also causes other types of tumor cells to secrete CEA. These observations led to the analyses of sera samples from patients treated with therapeutic doses of interferon to determine whether treatment could increase CEA or TAG-72 serum levels.

Sera were collected from 111 patients diagnosed with adenocarcinoma or nonadenocarcinoma malignancies and who received different schedules of interferon (IFN)-γ or IFN-βser alone or in combination. Serum carcinoembryonic antigen (CEA) and tumor-associated glycoprotein-72 (TAG-72) antigen levels were measured to determine whether interferon could enhance the tumor shedding and, thereby, the serum level of either tumor antigen. Less than 10% of the sera samples from patients diagnosed with nonadenocarcinoma malignancies (*e.g.*, hairy cell

leukemia, melanoma) had positive titers of TAG-72 or CEA, and interferon neither increased nor resulted in the appearance of either tumor antigen in those sera. In contrast, 59.2% and 75.4% of the patients with adenocarcinoma had positive serum levels of TAG-72 and CEA, respectively, prior to interferon. IFN-γ and IFN-βser alone or in combination significantly increased serum TAG-72 or CEA in approximately 65% of those patients. The results suggest that interferon administration to patients with adenocarcinoma can result in increased serum levels of selected tumor-associated antigens used in the diagnosis of malignancy. These preliminary findings may be important in the development of new strategies to obtain more sensitive tumor antigen serum assays for the diagnosis and monitoring for disease progression of adenocarcinoma.

Publications

Greiner JW, Guadagni F, Goldstein D, Borden EC, Ritts RE Jr, Lo Buglio AF, Suleh MN, Schlom J. Evidence for the elevation of serum carcinoembryonic antigen (CEA) and tumor-associated glycoprotein-72 (TAG-72) levels in patients administered interferon, Cancer Res 1991;51:4155-63.

Greiner JW, Guadagni F, Horan Hand P, Pestka S, Noguchi P, Fisher PB, Schlom J. Augmentation of tumor antigen expression by recombinant human interferons: Enhanced targeting of monoclonal antibodies to carcinomas. In Goldenberg, D. M. and McGuire WL (Eds.). Cancer Imaging with Radiolabeled Antibodies. Boston: Martinus Nijhoff 1990;413-32.

Guadagni F, Tortora G, Roselli M, Clair T, Cho-Chung YS, Schlom J, Greiner JW. Carcinoembryonic antigen regulation in human colorectal tumor cells by a site-selective cyclic AMP analogue: A comparison with interferon-gamma, Int J Cancer 1991;48:413-22.

Guadagni F, Witt PL, Robbins P, Schlom J, Greiner JW. Regulation of carcinoembryonic antigen expression in different human colorectal tumor cells by interferon-gamma, Cancer Res 1990;50:6248-55.

Shimada S, Ogawa M, Schlom J, Greiner JW. Identification of a novel tumor-associated M_r 110,000 gene product in human gastric carcinoma cells that is immunologically related to carcinoembryonic antigen, Cancer Res 1991;51:5694-703.

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

PROJECT NUMBER
Z01 CB 09018-08 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Anti-Carcinoma Monoclonal Antibody Clinical Trials

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

Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
Diane Milenic	Microbiologist	LTIB, DCBDC, NCI
Kathleen Siler	Microbiologist	LTIB, DCBDC, NCI
Diane Eggensperger	Senior Staff Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. J. Carrasquillo, CC, NM; Drs. B. Yu & C. Allegra, COP, DCT, NCI; Dr. E. Martin, Ohio State U., Dept. Surg.; Dr. S. Larson, Mem. Sloan-Kettering, Nucl. Med.; Dr. A. LoBuglio, U. of AL Med. Ctr., Dr. L. Murrry, M.D. Anderson Med. Ctr., Dr. K. Anderson, Dow Chem.

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SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.2	0.7	2.5

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☐ (a1) Minors
☐ (a2) Interviews

A

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

This project involves the use of monoclonal antibodies (MAbs) in both diagnostic and therapeutic clinical trials. To date, over 1,000 patients have been administered radiolabeled B72.3 in tumor-targeting studies carried out in numerous institutions, with similar findings of approximately 70-80% tumor targeting observed. The selective localization of 131-I MAb B72.3 IgG was demonstrated in biodistribution studies in colorectal cancer patients in which the percentage of injected dose of MAb per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% had an RI of at least 3 (*i.e.*, 3 times greater uptake per gram than normal tissues). We have also conducted studies to determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analysis of biopsy specimens).

A phase I therapy trial involving intraperitonel administration of 131-I-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity has been conducted. Extremely good localization of tumor lesions in the abdomen was detected. The use of a recombinant/chimeric MAbs has been conducted. Clinical trials involving the use of second and third generation MAb-isotope conjugate are in progress.

Major Findings

We have investigated the administration of radiolabeled MAb B72.3 IgG in a range of carcinoma patients. Several parameters were studied to determine factors that might influence MAb localization. No differences in MAb uptake were observed among lesions for numerous anatomic locations. No toxicity was observed. Radiolabeled B72.3 (anti-TAG-72) has been shown in numerous clincial trials to selectively localize metastatic lesions in 70-80% of the cases.

To assess the value of radioimmunoguided surgery in the intraoperative detection of ovarian cancer, we used MAb B72.3, radiolabeled with ^{125}I, and a hand-held gamma-detecting probe in 13 women with ovarian cancer undergoing exploratory laparotomy. B72.3 was injected 12-29 days preoperatively (intraperitoneally in four cases, intravenously in nine, and by both routes in one). Intraoperatively, the abdomen was surveyed with the probe and probe counts were correlated with biopsies and excised specimens studied by routine histologic stains. The specificity of the probe was 70%. Preoperative computed tomography was normal in all patients, including those with tumors as large as 3cm. This pilot study shows the ability of radioimmunoguided surgery to detect occult ovarian cancer.

Ten patients with peritoneal carcinomatosis received intraperitoneal injections of the murine monoclonal antibody B72.3 radiolabeled with ^{131}I (10 μCi, 0.8-1.0 mg of IgG), prior to surgery. The biodistribution of the radiolabeled antibody was determined using direct sampling of plasma, whole body counting, a dedicated nuclear medicine computer system, and a gamma camera. Laporatomy was performed 4-14 days after injection of radiolabeled antibody, and at the time of surgery, exploration and biopsies of normal tissues and tumor was performed, Histologic examination of the surgically excised tissues included an assay of the radioactivity content and immunohistochemistry for TAG-72, the tumor associated antigen recognized by B72.3. Tumor bound B72.3 had a biologic clearance half-time of 120 hours. B72.3 antibody which was not fixed to the tumor cleared from the peritoneal space with a half time of 30 hours. Whole body clearance was 72 hours. These studies permitted radiation dose estimates to normal organs and to tumor. Radioactivity which was cleared from the peritoneum and tumor was shown to be uniformly distributed in the whole body, and cleared through the kidney and bladder. Five source organs that were identified from the biologic studies were peritoneal cavity, intraperitoneal tumor, whole body, kidney and bladder. S-values were determined for radioactivity contained in the peritoneal cavity based on Monte Carlo methods and a newly developed geometric model of the peritoneal cavity developed at Oak Ridge Associated Universities. Also, the beta dose to the wall of the peritoneal cavity was computed.

Estimates of radiation doses to normal organs indicated that the marrow is likely to be the critical organ, and that the fraction of radiolabeled antibody bound to tumor affected the dose estimates. For example, for 100 μCi of B72.3 administered, computed marrow dose was 99 Rad at 0% retained in tumor and 122 Rad for 100% retained. Radiotoxicity may also be experienced for small intestine at these doses especially at very large fractions of antibody bound to tumor. (Rad dose to Small Intestine, 167 Rad (0% tumor bound) to 314 Rad (100% tumor bound) per 100 μCi ^{131}I B72.3 administered.)

Radiation dose varied directly with the concentration of radioactivity in tumor. The tumor radiation dose varied widely from site to site and from patient to patient with maximum radiation doses calculated at 11000 Rad per 100 μCi administered IP. Tumor implants received much higher doses than hematogenously derived metastases. There was a positive correlation between Rad dose and antigen content of tumors, but in a multivariate analysis it was evident that most of the variation was unexplained.

Considerable radiation is also delivered to the peritoneal surface from beta decay, but this falls off rapidly with distance and is important only to a depth of about 100 microns. However, particularly for microscopic, superficial disease tumoricidal doses of radiation are predicted.(*e.g.* in most superficial 30 microns of peritoneal surface, 6900 Rad/100 μCi delivered).

Taken together, these data suggest that at administered doses of radioactivity which are likely to retard tumor growth, normal tissue toxicity will not be life-threatening. Ideally, patients selected for this therapy should have relatively limited disease and good hematopoetic and renal function.

Chimeric B72.3, composed of the V-regions of murine B72.3 and the constant regions of human immunoglobulin G4 heavy and κ light chain was administered as a ^{131}I-labeled conjugate to 12 patients with metastatic colon cancer. Seven of these patients had an antibody response after initial infusion, and the immune response was primarily directed to the murine V-region, although a small proportion of the antibody response was directed to topographical epitopes requiring the presence of both murine V-region and human CH-I and κ constant regions (neo-epitopes). The pharmacokinetics included a plasma disappearance curve best fit by a two-compartmental model with an $t_{1/2}\alpha$ of 18±7 h and $t_{1/2}\beta$ of 224±66 h. A second infusion of the same dose of ^{131}I-chimeric B72.3 was administered to four of these patients 8 wk after the first infusion. Two patients who had a high antibody response to initial infusion had an anamnestic antibody response, and the infused ch-B72.3 rapidly disappeared from the circulation with associated immune complexes and free ^{131}I in the plasma. One patient with no initial antibody response had no antibody response and identical pharmacokinetics on second infusion. One patient with a modest transient antibody response to initial infusion had no antibody response on second infusion and a modest shortening of plasma circulation. Thus, the human immunoglobulin G4 isotype chimeric B72.3 monoclonal antibody has a plasma half-life 6 to 8 times as long as murine B72.3 and retains considerable immunogenicity in some patients which can adversely affect repetitive infusions.

Publications

Ferroni P, Milenic DE, Roselli M, Carrasquillo JA, Raubitschek A, Schlom J, Colcher D. Potential artifact for the increase of tumor associated antigens in serum samples from patients injected with monoclonal antibodies, Nucl Med Biol 1991;18:383-7.

Khazaeli MB, Saleh MN, Liu TP, Meredith RF, Wheeler RH, Baker TS, King D, Secher D, Allen L, Rogers K, Colcher D, Schlom J, Shochat D, LoBuglio A. Pharmacokinetics and immune response of ^{131}I-chimeric mouse/human B72.3 (human γ4) monoclonal antibody in man, Cancer Res 1991;51:5461-6.

Larson SM, Carrasquillo JA, Colcher D, Yokoyama Y, Reynolds JC, Bacharach SA, Pace L, Rotman M, Stabin M, Neumann RD, Sugarbaker P, Schlom J. Radiation dosimetry of intraperitoneally administered ^{131}I-radiolabeled B72.3 monoclonal antibody in patients with peritoneal carcinomatoses, J Nucl Med (in press).

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

PROJECT NUMBER
Z01 CB 09021-06 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Isolation and Characterization of Genes Coding for Carcinoma-Associated Antigens

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

Paul Robbins	Senior Staff Fellow	LTIB, DCBDC, NCI
Judy Kantor	Expert	LTIB, DCBDC, NCI
Scott Meissner	Staff Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.6	3.6	0

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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.)

Monoclonal antibodies (MAbs) have defined several antigens associated with human carcinomas. Two of the most widely studied antigens are carcinoembryonic antigen (CEA) and TAG-72. CEA is a 180 kD glycoprotein and TAG-72 is a high molecular weight mucin. Recent studies have demonstrated that CEA is a member of the immunoglobulin supergene family. These include CEA, normal cross-reacting antigen (NCA), biliary glycoprotein (BGP), and human pregnancy-specific beta 1-glycoprotein (SP1).

The use of anti-CEA MAbs for diagnosis and therapy have been explored by a number of labs. In order to assess the ability of CEA to serve as a target for active immunotherapy, a mouse model has been generated in our lab. Mouse tumor cell lines expressing high levels of CEA have been derived, and the characteristics of the expresssed gene products have been analyzed. These tumor cells have been shown to grow in immunocompetent mice, and in preliminaary experiments have shown to serve as targets for active immunotherapy, carried out with a CEA-vaccinia construct (see project # Z01 CB 09028-02).

Experiments in other systems have demonstrated the ability of purified proteins produced in a baculovirus system, to boost responses to recombinant proteins produced in vaccinia. Recently, experiments have begun to explore the use of the baculovirus system for production of recombinant CEA.

Major Findings

A mouse model system has been developed to study active and passive immunotherapy directed against CEA. Since mice do not express CEA, the MC-38 mouse colon adenocarcinoma cell line has been transduced with CEA. The MC-38 cell line forms tumors in syngeneic C57BL/6 mice. Several transduced clones were derived which express high levels of cell surface CEA were derived. The levels found on the surface were, in several clones, as high as those found on the surface of human colon carcinoma cell lines. Analysis of the product found in individual clones indicated that, whereas some expressed a 180 kD product similar to human CEA, others expressed truncated products. A 70 kD CEA variant was found to lack 2 of the 3 repeated domains present in the normal human CEA gene product. Nonetheless, this truncated product was found to express most of the MAb epitopes found on CEA.

Recent experiments have been carried out to generate recombinant CEA for use as an immunogen in the immunotherapy studies, as well as for epitope mapping studies. The baculovirus insect system was chosen, since high levels of recombinant proteins have been produced in this system. Recently, the complete CEA gene, as well as a number of constructs containing deletions, have been introduced in the baculovirus genome. The levels of protein production will be analyzed, and these products will be characterized for the expression of a number of epitopes of CEA.

Publications:

Robbins PF, Kantor J, Salgaller M, Horan Hand P, Fernsten P, Schlom J. Transduction and expression of the human carcinoembryonic antigen (CEA) gene in a murine colon carcinoma cell line, Cancer Res 1991;51:3657-62.

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

PROJECT NUMBER
Z01 CB 09025-05 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Antibody Directed Cellular Immunotherapy of Human Carcinoma

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

Kwong-Y. Tsang	Expert	LTIB, DCBDC, NCI
Rosaria DeFillippi	Fogarty Fellow	LTIB, DCBDC, NCI
Chen-Feng Qi	Fogarty Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. John Yanelli, Surgery Branch, DCT, NCI, NIH

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

We investigated the ability of human recombinant (hr) hrIL-6 to augment antibody directed cell mediated cytotoxicity (ADCC) via MAb D612 using colorectal carcinoma target LS-174T, WiDr and HT-29 cells. A significant increase in ADCC activity was observed after human PMNC were incubated in 100-400 U/ml of hrIL-6. hrIL-6 did not augment non specific (non MAb mediated) cytotoxicity. Enhancement of ADCC activity was blocked by the addition of an antibody against hrIL-6 but not by an antibody to the IL-2 receptor, suggesting the hrIL-6 augmentation of ADCC activity may not be mediated through IL-2. The surface antigen expression and susceptibility to lysis of human IL-6 gene transfected colorectal carcinoma cell line, HT-29 were investigated by flow cytometry and by 24 h In-111 release ADCC assay. We found that IL-6 transfected HT-29 cell can secrete a high level of biologically active IL-6. The expression of cell surface antigens in the transfected HT-29 cells were analyzed. Significant enhancement in the percent of CEA expressing cells as detected by COL-1 MAb but not in the percent of cells expressing HLA-class I, HLA class II, and ICAM-1 antigens as compared to the parental HT-29 cell was observed. The susceptibility to lysis of the transfected HT-29 cells increased significantly in ADCC assay using COL-1 MAb. The increase in ADCC activity correlated well with the increased expression of CEA. These results provide a rationale for use of IL-6 gene transfer into human cells as a possible modality for cancer therapy. We also investigated the tumoricidal properties of D612 MAb alone and in combination with IL-2 activated tumor lymphocytes in athymic mice bearing LS-174T colon tumor xenografts. The resutls demonstrate that the tumoricidal properties of LAK cells and the D612 MAb can be augmented when used together in immunotherapy of human colon cancer xenograft.

Major Findings

A monoclonal antibody (MAb), designated D612 (IgG2a), was generated by immunization of mice with a membrane enriched fraction of a moderately differentiated primary colon adenocarcinoma. Using membrane extracts derived from a variety of cell lines and normal and neoplastic tissues, D612 was found to react specifically with the extracts of cells or tissues originating from the large intestine. The D612 antigen was not detected by immunohistochemical staining of conventionally processed tissues specimens, but was revealed when fresh, frozen tissues were used. An immunohistochemical survey of the tissue distribution of the D612 antigen showed that 70 to 80% of primary or metastatic colorectal carcinomas were positive, independent of their differentiation. In over half of these specimens, the staining was homogeneously distributed among the majority of tumor cells where it localized predominantly to the plasma membrane. Flow cytometry of colon tumor cell lines also showed that the D612 antigen occupied a surface location. D612 did not stain noncarcinoma tumors but did react weakly with a small number of stomach, breast, and ovarian carcinomas in a highly focal pattern. Normal tissue reactivity of D612 was confined to the epithelium of the small and large intestine and of the stomach. It was not found in normal specimens covering a wide range of nongastrointestinal tissues including thyroid, kidney, pancreas, liver, bladder, blood cells, and lung. Normal colon goblet and absorptive cells showed a homogenous pattern of staining, located primarily along the basolateral cytoplasmic membrane. Other studies showed that the D612 antigen is not secreted into the culture media, has a molecular size of 1 x 10^6 or greater, and is not CEA, TAG-72 or the 17-1A antigen.

The D612 MAb was found to mediate antibody-dependent cellular cytotoxicity (ADCC) in conjunction with normal human PMNC against antigen-positive colon tumor cell lines. Exposure of PMNC to IL-2 (100 U/ml; 24 hr) resulted in a 2- to 3-fold increase in specific ADCC cytolytic activity. Although the total specific ADCC lytic activity varied among different donors, its potentiation by IL-2 was very similar. Optimal stimulation of specific ADCC with IL-2 appeared to be after 24 h of culture in 500U/ml of IL-2. Stimulation of ADCC was also seen at 10 U/ml of IL-2. Antibody dose titration with IL-2 treated or untreated effector cells indicated that the threshold dose of MAb required for efficient ADCC was reduced by 200 fold with IL-2. The majority of the ADCC activity were found associated with nonadherent cells. The results from depletion experiments suggest the participation of NK/LAK cells in the D612 mediated ADCC activity. The ADCC activity of the 17-1A MAb was also studied for comparison to D612, and it was found that both MAbs were similar in their ability to mediate ADCC.

Interleukin-6 (IL-6) can greatly increase the lytic activity of human LAK cells and can augment human natural killer cell activity. We have investigated the effects of human rIL-6 on ADCC activity of human PMNC using three distinct anti-colorectal carcinoma MAbs, D612, 17-1A and 31.1 to mediate ADCC activity. Significant increase in ADCC activity was observed after PMNC were incubated in 100 to 400U/ml of hrIL-6. The optimal ADCC activity was observed at a concentration of 100U/ml of hrIL-6. Variation of ADCC activities among effector cell donors were observed. Nonspecific MAb showed no effect in augmenting ADCC activity. hrIL-6 treatment did not augment nonspecific (non MAb mediated) cytotoxicity. Human rIL-6 augmented ADCC activity by 24 h after incubation of effector cells and was maximal at the 72 h incubation time (from 19% to 32%), hrIL-6 augmentation of ADCC was not observed at times earlier than 24 h. ADCC activity returned to the 24 h and 48 h level of augmentation at the 96 h incubation time. The enhancement of ADCC activity was blocked by the addition of an antibody to hrIL-6 but not by an antibody to the IL-2 receptor. Upon elimination of CD16+ cells by incubation with anti-leu 11b+c', treatment with IL-6 was unable to augment ADCC activity. These results suggest that the CD16+ subpopulation in PMNC is responsible for the ADCC activity as well as hrIL-6 augmentation of ADCC activity. To investigate if hrIL-6 could augment the induction of ADCC activity mediated by

PMNC exposed to suboptimal doses of hrIL-2, human PMNC were costimulated with 0, 10 or 100U/ml of hrIL-2 plus 0 or 100 U/ml of hrIL-6 for 24 h prior to ADCC assay. Pretreatment of PMNC with 100 U/ml of hrIL-6 augmented significantly the ADCC activity induced by 10U/ml of hrIL-2 (from 28% to 44%). However, the increase in ADCC in culture induced by 10 U/ml of hrIL-2 and 100U/ml hrIL-6 appears to be mainly due to an augmentation of LAK cell activity. ADCC activity was increased further when PMNC were costimulated with 100 U/ml of hrIL-2 and 100 U/ml of hrIL-6. At 100 U/ml of hrIL-2, hrIL-6 did not appreciably augment the LAK activity observed in cultures exposed to hrIL-2 alone. The results indicate a potential synergistic effect in the use of the combination of hrIL-2 and hrIL-6 in ADCC activity of human PMNC and suggests a potential role for IL-6 in combination with anti-cancer antibodies for cancer immunotherapy.

The tumoricidal properties of MAb D612 alone and in combination with IL-2-activated human lymphocytes were investigated in athymic mice bearing LS-175T colon tumor xenografts. Treatment of mice bearing LS-174T tumor (1 day s.c.) with i.v. dose of 400 μg of D612 alone resulted in a significant inhibition of tumor growth. Lower doses of D612 had an intermediate effect on tumor growth. Similar inhibition of tumor growth was obtained when D612 was administered in three doses of 400 or 800 μg each during the first week after tumor implantation. Human LAK cells were generated by incubating human peripheral blood mononuclear cells from normal donors with 100 U/ml of IL-2 for 24 h. An administration of human LAK cells did not significantly inhibit the growth of human xenograft tumors. Adoptive transfer of a single dose of human LAK cells (2×10^7 i.v.) into mice treated with a suboptimal dose of D612 (200 μg) significantly inhibited tumor growth compared to that obtained with three doses of D612 plus human LAK cells although there was a tendency for multiple doses of LAK cells alone to show some antitumor effects. LAK cells or PBL had similar antitumor activities when used in conjunction with D612. These results suggest that the tumoricidal properties of LAK cells and D612 MAb can be augmented when used together in the immunotherapy of human colon cancer xenograft.

The antigen expression and susceptibility to lysis of the HT-29 cells transfected with human interleukin-6 genes were also investigated by flow cytometry and by 24h ^{111}In release ADCC assay, respectively, using COL-1 MAb. The cDNA of the human IL-6 gene (580 bp) was inserted into a retroviral vector Hind III/Hpal linearized pLNCXII. Lipofection was used to introduce the expression construct into HT-29 cells. IL-6 activity was measured by ELISA and by bioassay. IL-6 secreted transfected HT-29 cell was biologically active. The expression of CEA, HLA class I, HLA class II and ICAM-1 antigens in the transfected HT-29 cells were analyzed. Significant enhancement in the expression of CEA as detected by COL-1 MAb but not in the expression of HLA class I, HLA class II and ICAM-1 antigens as compared to the parental HT-29 cells was observed. No susceptibility to lysis of the transfected HT-29 cells increased significantly in ADCC assay using COL-1 MAb. The increase in ADCC activity correlated well with the increase in the expression of the expression of CEA. Addition of anti-IL-6 receptor MAb (PM-1) did not block the increase in ADCC activity. Treatment of parental HT-29 cells with 100 U/ml of hrIL-6 for 24 h did not alter the expression of these antigens or change the susceptibility to lysis of the target cell in ADCC assay.

Publications

Tsang KY, Finch MD, Primus FJ, Schlom J. Human IL-6 enhances antibody dependent cellular cytotoxicity of human tumor cells mediated by human peripheral blood mononuclear cells mediated by human peripheral blood mononuclear cells, Cancer Immunol Immunother 1991;34:9-16.

Pendurthi TK, Schlom J, Primus FJ. Human Lymphokine activated killer cells augment immunotherapy of human colon carcinoma xenografts with monoclonal antibody D612, J Immunother 1991;10:2-12.

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

PROJECT NUMBER
Z01 CB 09028-02 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Active Immunotherapy to Human Carcinoma Associated Antigens

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

Judy Kantor	Expert	LTIB, DCBDC, NCI
Paul Robbins	Senior Staff Fellow	LTIB, DCBDC, NCI
Judy DiPietro	Biologist	LTIB, DCBDC, NCI
Scott Abrams	Senior Staff Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
10.2	4.7	4.5

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☐ (a1) Minors
☐ (a2) Interviews

A

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

Active specific immunotherapy is a new and potentially non toxic approahc for cancer therapy. Tumor associated antigens could serve as targets for this type of therapy. Carcinoembryonic antigen (CEA) is a 180,000 dalton oncofetal glycoprotein expressed on most gastrointestinal carcinomaas and many other human carcinoma types. CEA is generally considered to be weakly immunogenic in humans; that is, little evidence exists for humoral or cell mediated immunity to CEA in normal or cancer patients. The copresentation of CEA with a strong immunogen would represent a logical approach to inducing anti-CEA responses for tumor immunotherapy. Recent advances in recombinant vaccinia virus technology has provided a powerful method for antigenic copresentation. To this end, a 2.4 bb cDNA clone, containing the complete coding sequence, was isolated froom a human colon tumor cell library and was inserted into a vaccinia virus genome. This recombinant construct was characterized by Southern blotting, restriction endonuclease digestion, polymerase chain reaction analysis, and subsequent DNA hybridization. The CEA gene was stably integrated in the vaccinia virus thymidine kinase gene. This recombinant was efficiently replicated upon serial passages in cell cultre and in animals. The recombinant virus expressed on the surface of infected cells, a protein product recognized by the MAb COL-1 directed against CEA. Immunizaation of mice with the recombinant vaccinia virus resulted in a humoral immune response against CEA. Pilot studies demonstrated that the administration of the recombinant vaccinia virus was able to greatly reduce the growth of tumors in mice. This murine colon adenocarcinoma had been transduced with the human CEA gene, expressed human CEA, and grew in syngenic animals. The use of this new recombinant CEA vaccinia virus may provide a new approach in the specific active immunotherapy of human gastrointestinal cancer and other CEA expressing carcinoma types.

Major Findings

Carcinoembryonic antigen (CEA) is one of the most widely studied oncofetal tumor-associated antigens. Although CEA is not specific for colon cancer, CEA has clinical utility in the surveillance of the post-operative patient following primary tumor resection. The development of monoclonal antibodies (MAbs) directed against CEA has led to improvements in the diagnostic imaging of primary colon tumors and the immunolocalization of metastatic disease. Several more recent approaches, including anti-idiotypic antibodies and radiolabeled and drug conjugated MAb, have prompted interest in targeting CEA for immunotherapy. CEA is generally considered to be weakly immunogenic in humans; that is, no evidence exists for humoral or cell-mediated immunity to CEA in normal or cancer patients. Therefore, the co-presentation of CEA with a strong immunogen would represent a logical approach to inducing an anti-CEA response for tumor immunotherapy. Recent advances in recombinant vaccinia virus technology provide a powerful method for such antigenic co-presentation. Vaccinia virus is highly immunogenic and stimulates both humoral and cell mediated immune responses; this cell mediated immunity may be especially important in tumor rejection. Vaccinia virus is also capable of presenting tumor antigens along with cellular major histocompatibility antigens. Thus, immunization with vaccinia virus provides a strong stimulus to the immune system.

The introduction of foreign genes into vaccinia virus has been possible with recent advances in molecular biology and genetic engineering. Vaccinia virus recombinants are generally stable and properly replicate and transcribe foreign genes inserted under the regulation of a vaccinia virus promoter. The CEA cDNA was inserted into the PSC-11 plasmid behind the vaccinia virus promoter p7.5. The plasmid construct was then inserted into the vaccinia virus genomes by homologous recombination. Viral particles were collected and plated onto monolayers of TK^-143 B cells in the presence of BuDR and X-Gal. Blue plaques were isolated and purified by several rounds of dilution and selection. After purification, recombinants were characterized by the comparison of a Southern blot containing a HindIII digest of wild type vaccinia virus DNA with that of the recombinant viral DNA. The blot was hybridized to a vaccinia virus probe and the recombinant DNA clearly lacked the 5.1 Kb HindIII J fragment when compared to the wild type DNA, indicating that the CEA gene was inserted into the TK gene of the vaccinia virus. The size of the recombinant HindIII J fragment, now containing the human CEA gene, the Lac Z gene and part of the viral TK gene, was determined by hybridization with a Lac Z probe. The probe detected a 9.2 Kb band in the recombinant DNA lane but not in the wild type lane. This is constant with the expected size of the recombinant HindIII J fragment. DNA hybridization studies were performed to show that the recombinant virus contained the CEA gene. Recombinant and wild type virus were plaqued and the DNA was directly transferred to nylon membranes and hybridized to a CEA radiolabeled probe. Recombinant vaccinia virus plaques hybridized to the CEA probe whereas the wild-type vaccinia plaques did not. Several recombinant clones were analyzed by PCR to show the presence of the full length CEA gene. DNA samples taken from plaques were collected, treated with 30 cycles of amplification followed by gel electrophoresis, transferred to a nylon membrane and hybridized to a radiolabeled CEA probe. A 2.1 Kb band was amplified in the recombinants only indicating the entire 2.1 Kb CEA gene was in the recombinant vaccinia virus. The expression and cellular localization of CEA protein was determined by immunofluorescent staining with monoclonal antibody COL-1 directed against CEA. The cells infected with the recombinant vaccinia virus construct showed distinct cell surface staining with the monoclonal antibody COL-1 under fluorescence.

The recombinant vaccinia virus expresses CEA and is able to insert the molecule in the cellular membrane consistent with the normal cellular localization of CEA. Cells infected with wild-type vaccinia virus failed to show any immunofluorescent staining with COL-1. Furthermore, immunofluorescent staining with the isotype matched negative control antibody B72.3 failed to elicit any imaging on cells infected with the recombinant vaccinia virus containing CEA.

The recombinant vaccinia virus construct was used to immunize C57BL/6 mice 3 times with 10^8 PFU of virus at 2 week intervals by intraperitoneal injection. The mice developed antibody titers to CEA within 14 days of inoculation as determined by an ELISA assay using purified CEA as antigen. A control group of mice were also inoculated with 10^8 PFU of wild type vaccinia at two week intervals. Anti vaccinia antibodies were detected by ELISA, however, there was no detection of any CEA antibody in these mice. This data suggests that mice immunized with the recombinant virus containing CEA can recognize human CEA and mount a humoral immune response against this antigen. None of the vaccinated mice exhibited any evidence of toxicity for the 42 day observation period following immunization.

Pilot studies were also conducted to determine if there was any biologic activity to the recombinant CEA vaccinia construct. Human CEA was transduced into and expressed in the MCA 38 murine colon adenocarcinoma cell line. These CEA-transduced cells were shown to grow as subcutaneous tumors in syngenic C57BL/6 mice. 1×10^6 MCA 38 transduced cells were transplanted into C57BL/6 mice. Seven days later, the mice were given three administrations of either wild type vaccinia virus or recombinant vaccinia virus 14 days apart. Animals that had been administered the recombinant vaccinia virus containing human CEA experienced a dramatic reduction in the size of the tumors during the course of 42 days. Two of the animals that received the recombinant vaccine never developed tumors. In contrast, animals administered wild type virus failed to stop tumor growth. Animals receiving no vaccinia also developed tumors and their growth rate was similar to that of the animals administered wild type vaccinia.

We plan to use the human tumor associated antigen, CEA, as a target molecule for the development of vaccines in specific active immunotherapy protocols. We will compare the efficacy of tumor rejection in animal models using recombinant vaccinia virus vaccinations with immunization schedules using purified human CEA protein, CEA specific peptides and anti-idiotype antibodies. We will define the Immunological mechanisms as well as the immunodominant epitopes on CEA which might be involved in tumor rejection in this model.

Publications

Kaufman H, Schlom J, Kantor, J. A recombinant vaccinia virus expressing human carcinoembryonic antigen (CEA), Int J Cancer 1991;48:900-7.

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

PROJECT NUMBER
Z01 CB 09022-06 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cytoskeletal Proteins in Oncogenic Transformation and Human Neoplasia

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

Herbert L. Cooper	Chief, Cell. & Molec. Physiol. Section	LTIB, DCBDC, NCI
Gaddamanugu Prasad	Visiting Fellow	LTIB, DCBDC, NCI
Rebecca Fuldner	Senior Staff Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

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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 have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous observations have led us to hypothesize that: a.) TM suppression is a causal event in neoplastic transformation; and b.) the oncogenic pathways initiated by a number of different oncogenes and other modalities converge on and act through TM suppression. We have obtained evidence supporting this hypothesis by restoring expression of TM1, one of two suppressed tropomyosins in the v-Ki-*ras* transformed NIH3T3 cell line, DT, thorugh the use of a retroviral vector (pBNC) carrying a full length cDNA encoding human TM1. Cell clones expressing the cDNA had elevated levels of TM1 and lost the ability to grow under anchorage-independent conditions. They also did not participate in formation of tumors in athymic mice. The ability of elevated levels of TM in the transduced clones to be utilized in the cytoskeleton is under study, as is their participation in the formation of the physiological dimers in which TM occurs *in vivo*. Complete reversal of the transformed phenotype may require simultaneous restoration of both TM1 and TM2 to permit formation of hereodimeric TM molecules, which are the preferred form *in vivo*. Materials for the insertion of TM2-encoding cDNA into clones already restored in TM1 expression are now being prepared. TM1 expression has also been restored in the human breast cancer cell line, MCF-7, which we previously showed to be defective in synthesis of this TM isoform. Testing for effects of this restoration on the neoplastic phenotype is currently in progress.

Major Findings

We have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous work has established that expression of two higher M_r TMs (TM1 and TM2) was consistently reduced or eliminated in murine cells transformed by a variety of retroviral oncogenes, DNA tumor viruses, chemical carcinogens, or transforming growth factor α (TGF-α). We have also examined a number of commonly studied cell lines derived from human breast carcinomas and found them all to be defective in expression of TM1, together with other abnormalities of TM expression. This suggests that tropomyosin suppression may play a role in human carcinoma. These observations have led us to hypothesize that:

a. TM suppression is a causal event in neoplastic transformation; and

b. the oncogenic pathways initiated by a number of different oncogenes and other modalities converge on and act through TM suppression.

To test this hypothesis, we have inserted a full-length cDNA encoding human TM1 into *v-Ki-ras* transformed NIH3T3 cells (DT cell line), in which both TM1 and TM2 are downregulated. Insertion was accomplished by means of a retroviral vector (pBNC). Cloned cell lines (S-clones) were obtained which expressed large amounts of TM1 derived from the inserted cDNA. These clones lost the ability to grow as colonies in semi-solid agar. On subcutaneous injection into athymic mice, S-clone cells produced tumors after a 3-5 day delay, compared with controls. Northern blot analysis showed that tumors developing from S-clone inocula no longer expressed the inserted TM1 cDNA, indicating that cells expressing elevated amounts of TM1 did not participate in tumor formation. In 2 of 3 cases, S-clone tumor cells, when re-cultured, again expressed the inserted TMe1 cDNA. Thus, some factor in the *in vivo* milieu suppressed expression of the TMe1 insert. This was apparently a specific effect, since neomycin phosphotransferase activity was still present in S-clone tumors. The gene for this enzyme (neo^r) was inserted as part of the pBNC vector and is responsible for the G418 resistance used as a selectable marker. These results indicate that TM suppression is necessary for two components of the transformed phenotype that closely correlate with neoplastic potential, namely, anchorage independent growth and the ability to participate in tumor formation in athymic mice. Moreover, they demonstrate that these neoplastic features are susceptible to reversal by molecular replacement of TM1 expression.

Other aspects of the transformed phenotype were not as prominently affected by TM1 replacement. Immunofluorescence studies of microfilament structures showed that the marked disruption of microfilament bundles characteristic of DT and other transformed fibroblast lines was only partially restored by elevation of TM1 expression. S-clones still exhibited multilayered growth in monolayer culture and were, thus, not typical 'flat' revertants.

The explanation for this partial effect of TM1 restoration may lie in the continued suppression of TM2 expression in S-clone cells. TM occurs *in vivo* as a side-to-side dimer which polymerizes end-to-end along the actin microfilament. Studies with smooth and skeletal muscle cells have shown that the thermodynamically preferred dimer is a heterodimer (e.g.: TM1:TM2) which also participates better than homodimers in end-

to-end polymerization in microfilaments. Since TM2 expression is virtually absent in DT cells and S-clones, the elevated synthesis of TM1 in S-clones may be expected to produce large numbers of TM1 homodimers which might not be optimally utilized in the formation of cytoskeletal structures. We found, in fact, that cytoskeletal utilization of TM1 in S-clone cells, while much increased over that in DT cells, was only half the level found in normal NIH3T3 cells. Studies of TM dimer formation, by a disulfide cross-linking technique, confirmed that the high levels of TM1 were present in S-clone cells as homodimers. These homodimers were found to enter the cytoskeleton, but, as noted above, in amounts below normal levels. Since homodimers may form end-to-end polymers poorly, they may function inadequately as part of the microfilament and therefore fail to produce full suppression of the transformed phenotype.

To test this possibility, we have produced a cDNA encoding the murine TM2 isoform. This cDNA is currently being cloned into an expression vector, and will shortly be inserted by transfection into S-clone cells already expressing high levels of TM1 but little TM2. We anticipate obtaining cell lines, originally derived from DT and still expressing v-Ki-*ras*, but expressing high levels of both TM1 and TM2. Such lines will be tested for various components of the transformed phenotype, and TM utilization and dimer formation will be analyzed.

To investigate the basis for specific suppression of TMe1 expression in vivo, we have produced a modified TM1 cDNA lacking nearly all of the 5' and 3' untranslated regions of the normal mRNA. With this construct we will test the hypothesis that suppressive regulatory elements in these untranslated regions may be targeted by factors induced by the *in vivo* milieu.

In order to extend our studies of the effect of TM restoration to human cancers, which arise primarily in epithelial cells, we have inserted TMe1 cDNA into the MCF-7 line of human breast cancer cells. We showed previously that these cells lack expression of TM1, and of a 38 kDa TM specific to epithelial cells. We have now obtained several cell clones (MCF-7/T1 clones) expressing high levels of TM1. Tests of the effect of TM1 restoration on the neoplastic phenotype are in progress.

In the course of screening a human mammary epithelial cell (strain 184) cDNA library for epithelial cell-specific human tropomyosins, a full length cDNA clone a 2s kDa protein, which was named HME1, was isolated. On sequencing, this clone was found to be unrelated to tropomyosin. Expression of HME1 RNA appears to be limited to epithelial cells. By northern blot analysis, it was found to be expressed by epithelial derivatives of all three embryonic layers (ectoderm, endoderm, mesoderm) but not by any non-epithelial derivatives (fibroblasts, lymphocytes, neuroglia, melanoma). The HME1 sequence has extensive sequence homology with bovine 14-3-3 protein, which is an activator of tyrosine and tryptophan hydroxylase. However, the tissue distribution, arrangement of charged amino acids, and location of potential phosphorylation sites of HME1 differ from those of 14-3-3. HME1 is therefore likely to be a member of a gene family which includes 14-3-3 protein. Compared with normal mammary epithelial cells, expression of HME1 RNA was dramatically low in two cell lines derived from human mammary carcinoma that were examined (MCF-7 & T47D), and in A1N4/TH cells, which are the immortalized, nontransformed human mammary epithelial cell line, 184A1N4, transformed by the combined action of the polyoma T gene and the Ha-*ras*

oncogene. HME1 therefore appears to be a cellular differentiation marker that may be down-regulated during neoplastic transformation. Specific antisera are now being raised which may be useful in tissue diagnosis and detection of neoplastic change.

Future plans

Future plans for this project include cloning and expression of TM2 in DT cells and in DT/S clones already enhanced in TM1 expression; similar manipulations of human mammary carcinoma cell lines; study of the basic mechanism of TM suppression by oncogene expression; study of the relationship of TM suppression to microfilament disruption and to the neoplastic phenotype; development of modified retroviral vectors that may be targeted to specific cell types; development of TM isoform specific antisera and monoclonal antibodies to screen human tumor sections for detection of abnormalities of TM expression.

Publications

Cooper HL, Fuldner R, McDuffie E, Braverman R. T-cell receptor activation induces rapid phosphorylation of prosolin which mediates down-regulation of DNA synthesis in proliferating peripheral lymphocytes, J Immunol 1991;146:3689-96.

Prasad GL, Meissner S, Sheer DG, Cooper HL. A cDNA encoding a muscle-type tropomyosin cloned from a human epithelial cell line: identity with fibroblast tropomyosin TM1, Biochem Biophys Res Commun 1991;177:1068-75.

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

PROJECT NUMBER
Z01 CB 09003-10 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Role of EGF-related Peptides in the Etiology & Progression of Breast & Colon Cancer

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

David S. Salomon	Chief, Tumor Growth Factor Section	LTIB, DCBDC, NCI
Nicola Normanno	Visting Fellow	LTIB, DCBDC, NCI
Robert Callahan	Chief, Oncogenetics Sections	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. Marc Lippman, Dir., Lombardi Cancer Ctr., Georgetown Univ., Washington, DC.; Dr. George Todaro, Bristol-Meyers Squibb, Seattle, WA.

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Tumor Growth Factor Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.7	3.7	2.0

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☐ (a1) Minors
☐ (a2) Interviews

B

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

Transforming growth factor α (TGFα), amphiregulin (AR) and cripto 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. TGFα has been 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 TGFα and interference with its biological activity have not been thoroughly examined; and relative levels of expression and biological function of AR and cripto in these malignacies are unknown. Present studies demonstrate that transformation of human mammary epithelial cells with a point-mutated c-Ha-*ras* protooncogene, but not with a c-*erb*B-2 oncogene, results in an increase in TGFα expression. Also, overexpression of human TGFα cDNA in these cells leads to *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 *ras* or TGFα transformed mammary cells, suggesting the establishment of an external autocrine loop. Estrogens can increase TGFα mRNA and protein expression in estrogen-responsive human breast cancer cell lines like MCF-7 cells. Transient transfection assays in MCF-7 cells using a plasmid containing the TGFα promoter ligated to either the chloramphenicol acetyltransferase (CAT) or luciferase genes demonstrate that physiological concentrations of estrogens can induce a 10-to 100-fold increase in the activity of these reporter genes. This suggests that the TGFα promoter contains a *cis*-acting estrogen-responsive element (ERE). MCF-7 cells were infected with a recombinant amphotropic TGFα antisense expression vector. Expression of this antisense RNA leads to partial reduction in basal and estrogen-induced TGFα protein production and to an equivalent degree of inhibition of basal and estrogen-induced proliferation. Specific mRNA transcripts for AR and cripto were detected in ~70% of primary and metastatic colorectal tumors, but only 5% of normal colon or liver tissue expressed these genes. In contrast, cripto mRNA was not expressed in either normal or malignant human mammary tissue whereas AR mRNA was found in ~ 50% of these samples.

Major Findings.

We have demonstrated that TGFα is consistently overexpressed 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 oncogene transformed mammary epithelial cells. Spontaneously immortalized MCF-10A normal human mammary epithelial cells can be transformed after transfection with an activated human c-Ha-*ras* protooncogene or with an activated c-*erb* B-2 oncogene. Both *ras* and *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 anchorage-dependent growth (ADG) rate in serum-free medium that is devoid of EGF. In the *ras* and *erb* B-2 transformed MCF-10A cells there is a reduced mitogenic responsiveness to exogenous EGF. In *ras* transformed cells, but not in the *erb* B-2 transformants, there is a 4- to 8-fold increase in the level of TGFα mRNA expression and TGFα protein production suggesting that TGFα is involved in the transformation of mammary epithelial cells by an activated c-Ha-*ras* gene, but not by the *erb* B-2 gene. MCF-10A cells were infected with an amphotropic retroviral vector containing the human TGFα gene to ascertain the transforming potential of this gene. Overexpression of this vector leads to a 15- to 20-fold increase in the production and secretion of TGFα. These TGFα overexpressing mammary epithelial cells form colonies in soft agar, exhibit an enhanced growth rate in serum-free medium and show a diminished response to exogenous EGF. Growth of the *ras* or TGFα transformed cells in soft agar can 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. Similar results have been observed in HC11 mouse mammary epithelial cells. These cells can be induced to differentiate in response to lactogenic hormones such as prolactin and glucocorticoids after which they synthesize β-casein. HC11 cells transformed with an activated human Ha-*ras* protooncogene or TGFα gene are no longer able to differentiate in response to lactogenic hormones whereas *erb* B-2 transformed HC11 cells are still able to synthesize β-casein in response to these hormones. Addition of an anti-EGFR blocking antibody is able to restore the ability of the *ras* and TGFα transformed cells to respond to lactogenic hormones suggesting that secreted TGFα is acting through an autocrine mechanism to negatively regulate β-casein expression through the EGF receptor. In addition, the data suggest that activation of the EGF receptor by TGFα and of the *erb* B-2 receptor by an unidentified ligand(s) has different effects upon mammary epithelial cell differentiation. TGFα and other growth factors may also be elaborated by stromal cells and thereby influence the behavior of adjacent mammary epithelial cells that have been sensitized to these growth factors in a paracrine manner. For example, we have found that human mammary epithelial cells which overexpress c-*myc* can form colonies in soft agar in response to EGF, TGFα or basic FGF. Likewise, co-culivation of these *myc* expressing cells with primary human diploid mammary fibroblasts can also induce their AIG in soft agar. Conditioned medium (CM) obtained from the fibroblasts can mimic this effect. CM from these cells contains biologically active and immunoreactive TGFα and basic FGF and the fibroblasts express basic FGF mRNA.

The expression of TGFα mRNA and TGFα protein in estrogen receptor (ER) positive human breast cancer cells such as MCF-7 or ZR-75-1 can be increased by growth-promoting concentrations of 17β-estradiol (E2) whereas in ER negative breast cancer cell lines such as MDA-MB-231 cells basal levels of TGFα are generally higher than in the ER positive cell lines and insensitive to E2 regulation. To ascertain if E2 can directly regulate TGFα expression through the TGFα promoter, MCF-7 and ZR-75-1 cells were transiently transfected with plasmids containg the TGFα promoter ligated to either the chloramphenicol acetyltransferase

(CAT) or luciferase reporter genes. MCF-7 or ZR-75-1 cells transfected with either plasmid and subsequently treated with physiological concentrations of E2 (10^{-10} M to 10^{-7} M) for 24 hrs exhibited a 10- to 100-fold increase in either CAT or luciferase activity. This induction by E2 could be blocked by simultaneous treatment of the cells with the antiestrogens, tamoxifen or droloxifen. E2 was unable to affect CAT or luciferase activity following transfection of these reporter plasmids into MDA-MB-231 cells. To ascertain if E2-induced proliferation could be attenuated by blocking the expression of endogenous TGFα, MCF-7 or ZR-75-1 cells were infected with an amphotropic retrovirus containing the TGFα gene in the 3' to 5' orientation in order to generate a specific antisense mRNA. Infected MCF-7 or ZR-75-1 cells exhibited a 50% to 60% reduction in E2-stimulated TGFα production and a 45% to 70% reduction in ADG or AIG after induction of the antisense vector. In primary human breast tumors, an association exists between high EGF receptor expression and an ER negative phenotype. 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 sites/cell, were transfected with an expression vector plasmid containing the human EGF receptor gene and a selectable *neo* marker. Several *neo* resistant ZR-75-1 clones were selected and found to express over 1.2×10^6 EGF receptor sites/cell. These overexpressing clones possessed functionally normal EGF receptors since they could be autophosphorylated in response to exogenous EGF and could transphosphorylate the p185 *erb* B-2 protein in these cells. No change in the number or affinity of ER were observed in these clones. More importantly, E2 was still capable of stimulating the ADG and AIG of these clones demonstrating that an increase in EGF receptor expression may be necessary but is not sufficient to induce an estrogen-independent phenotype.

TGFα is one of several EGF-related proteins that may be involved in regulating the proliferation of tumor cells through an autocrine mechanism. AR and cripto are two other members of this family and AR can bind to the EGF receptor. Specific mRNA transcripts for TGFα (4.8kb), AR (1.4kb) and cripto (2.2kb) are expressed in a majority of human colon cancer cell lines. Cripto and AR are also expressed in 60% to 70% of 78 primary or metastatic colorectal tumors, whereas only 2% to 7% of 38 normal noninvolved colon tissues or normal liver expressed these transcripts. Immunolocalization studies demonstrated that AR protein can be detected in the colorectal tumor cells and not in the surrounding stroma or noninvolved colonic epithelium. AR but not cripto is also expressed in both normal and malignant breast tissues. Approximately 50% of reduction mammoplasty samples and primary breast tumors express AR mRNA.

Publications

Ciardiello F, Dono R, Kim N, Persico MG, Salomon DS. Expression of cripto, a novel gene of the epidermal growth factor gene family, leads to *in vitro* transformation of a normal mouse mammary epithelial cell line, Cancer Res 1991;51:1051-4.

Ciardiello F, Gottardis M, Basolo F, Pepe S, Normanno N, Dickson RB, Bianco RA, and Salomon DS. Additive effects of c-*erb* B-z, c-Ha-*ras* and transfoming growth factor a genes on the *in vitro* transformation of human mammary epithelial cells. Mol Carcinogenesis (In press).

Ciardiello F, Kim N, Saeki T, Donas R, Persico MG, Plowman GD, Garrigues J, Radke S, Todaro GJ, Salomon DS. Differential expression of epidermal growth factor-related proteins in human colorectal tumors, Proc Natl Acad Sci USA 1991;88:7792-6.

Colletta G, Cirafici AM, DiCarlo A, Ciardiello F, Salomon DS, Vecchio G. Constitutive expression of transforming growth factor a rat thyroid-epithelial cells, Oncogene 1991;6:583-7.

Johnson GR, Saeki T, Aversperg N, Gordon AW, Shoyab M, Salomon DS and Stromberg C. Response to an expression of amphiregulin ovarian carcinoma and normal ovarian surface epithelial cells: Nuclear localization of endogenous amphiregulin, Biochem Biophys Res Commun 1991;180:481-8.

Kuniyasu H, Yoshida K, Yokosaki H, Yasui W, Ito H, Toge T, Persico MG, Saeki T, Salomon DS, Tahara E. Expression of cripto, a novel growth factor in human gastrointestinal carcinomas, Jap J Cancer Res 1991;82:969-73.

Saeki T, Cristiano A, Lynch MJ, Brattain M, Kim N, Normanno N, Kenney N, Ciardiello F, Salomon DS. Regulation by estrogen through the 5'-flanking region of the transforming growth factor α gene, Mol Endo 1991;5:1955-64.

Venesio T, Taverna, D, Hynes N, Deed R, MacAllan D, Ciardiello F, Valrecins E, Salomon, DS, Callahan R, Merlo G. The *int*-2 gene product acts as a growth factor and substitutes for basic fibroblast growth factor in promoting the differentiation of a normal mammary epithelial cell line, Cell Growth and Differentiation 1991;3:63-71.

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

PROJECT NUMBER
Z01 CB 04829-17 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Identification and Characterization of Human Genes Associated with Neoplasia

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

Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBDC, NCI
Giorgio Merlo	Visiting Associate	LTIB, DCBDC, NCI
Craig Cropp	Senior Staff Fellow	LTIB, DCBDC, NCI
Zong-mei Sheng	Visiting Fellow	LTIB, DCBDC, NCI
Francesca Diella	Guest Worker	LTIB, DCBDC, NCI
Shikichi Miyazaki	Visiting Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. R. Lidereau, Centre Rene Huguenin, St. Cloud, France; Dr. D. Liscia, S. Giovanni Hospital, Torino, Italy; Dr. G. Campbell, Lab. of Stats. and Math. Methodology, NIH; Dr. J. Minna, NCI; Dr. L. Liotta, NCI; Dr. R. White, Howard Hughes Med. Ctr., U. of Utah.

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Oncogenetics Section

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

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.)

The etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken an ongoing program aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association with the patients history, characteristics of the tumor, and the patients prognosis. The most frequent type of mutation is loss of heterozygosity (LOH) at specific regions of the cellular genome in tumor DNA. In previous studies, we have found LOH on chromosomes 1p, 1q, 3p, 11p, 13q, 17p, 17q, and 18q. Our current results demonstrate LOH of the c-met proto-oncogene on chroomoosooome 7q in 40.5% of the tumor DNAs. This genetic alteration was not associated with the lymph mode status of the patient nor other standard prognostic features of the tumor. However, patients having tumors with LOH on chromosome 7q had a significantly shorter disease-free interval (p=0.00022) and overall survival (p=0.0036). A separate panel of 96 primary breast tumors was evaluated for their proliferative index by their ability to incorporate BruD in culture. A significant association (p-0.022) was found between those tumors having an elevated BruD labeling index and LOH at the pYNZ22.1 locus on chromosome 17p. In constrast, no association was found between the tumor BrdU labeling index and LOH at the more telemeric locus p144D6 on chromosome 17p nor LOH on chromosomes 1p, 3p, 13q, or 18q. To determine whether the p53 tumor suppressor gene is a target for LOH on chromosome 17p we examined 26 tumors by RNAse protection assays and nucleotide sequence analysis for p53 mutations. The same three tumors were found to have point mutations in the p53 gene. We also did single strand conformation polymorphism (SSCP) analysis which proved more sensitive in the detection of mutations. Taken together, the results showed that a total of 12 p53 mutations in 11 tumors (46%). Currently we are determining whether p53 mutations are linked to the proliferative index of the tumor.

Major Findings:

We have continued our systematic analysis of the human genome in a panel of 189 primary invasive ductal carcinomas of the breast. Our approach has been to use multiple recombinant DNA probes which detect restriction fragment length polymorphisms (RFLP) at regular intervals along each chromosomal arm. The most frequent type of mutation is loss of heterozygosity (LOH) at specific regions of the human genome. LOH is recognized as evidence for a tumor-suppressor gene located within the corresponding region of the homologous chromosome. During the course of our analysis we have found allelic loss at the c-*met* proto-oncogene on chromosome 7q31 in 40.5% of the tumor DNAs from 121 informative (heterozygous) patients. The c-*met* proto-oncogene encodes a tyrosine kinase which has recently been shown to be the cell-surface receptor for the hepatocyte growth factor. LOH of c-*met* was not significantly associated with standard prognostic features including tumor size, histopathological grade, and lymph node or steroid receptor status. However, patients with LOH on chromosome 7q31 in tumor DNA had a significantly shorter metastasis-free survival ($p=0.00022$) and overall survival ($p=0.0036$). These findings indicate that this region of chromosome 7q may harbor a breast tumor or metastasis suppressor gene, whether c-*met* is the target or some closely linked gene remains to be determined.

The capacity of tumor cells to proliferate might be associated with specific genetic mutations in the primary tumor. To test this hypothesis a panel of 96 breast carcinomas for which the BrdU labeling index was known, were analyzed for LOH on several of the chromosomes which we previously had shown in another tumor panel were frequently affected by LOH. We found that LOH on chromosome 17p at the pYNZ22.1 was significantly associated with tumors having an elevated BrdU labeling (proliferation) index ($p=0.022$). In contrast, no significant association was found between BrdU labeling index and LOH at the more telomeric p144D6 locus on chromosome 17p nor on chromosomes 1q, 3p, 13q, or18q. These data are consistent with the presence of a gene or genes on chromosome 17p13 near the pYNZ22.1 locus whose normal functioning is necessary for controlling breast tumor cell proliferation in vivo. Since earlier studies by others have shown that the proliferative index of a breast tumor is an independent predictor of disease-free interval and overall survival, it seems possible that mutation of the putative target gene(s) could also be predictive of disease outcome. At the present time however this tumor panel is not "mature" enough (3-4 year follow-up) to make that determination.

We have begun a comprehensive molecular analysis of the short arm of chromosome 17 to further define the location and identity of the putative tumor suppressor gene(s). The p53 gene is located on chromosome 17p13 centromeric to the pYNZ22.1 locus and is frequently altered by point mutation and deletion in a variety solid tumors. The p53 gene product is a nuclear phosphoprotein which appears to have an important negative role in regulating the transition from G1 to S phase of the cell cycle. Thus it seemed a likely candidate target gene for mutations which are associated with tumors having a high proliferative index. We have completed a systematic analysis of 26 primary breast tumors for mutations in the p53 gene by an RNAse protection assay and nucleotide sequence analysis of PCR-amplified p53 complimentary DNAs. Each method detected p53 mutations in the same three tumors. One tumor contained two mutations in the same allele. Single strand conformation polymorphism (SSCP) analysis of genomic and complimentary DNA proved more sensitive in the detection of mutations. Combining this technique with the other two a total of 12 mutations in the p53 gene were found in eleven tumors (46%). Not all of the tumors containing p53 point mutations had LOH of the remaining allele, suggesting that LOH may represent a later event. In addition, not all tumors having LOH at pYNZ22.1 or p144D6 had a point mutation at p53. Currently we are expanding this data base to determine whether p53

mutations are linked to tumors having a high proliferative index and whether there may be yet another tumor suppressor gene more closely linked to pYNZ22.1 that is also associated with the proliferative index of the tumor.

Publications:

Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa APM, Chiba I, Takahashi T, Nau MM, Callahan R, Minna JD. Mutations in the p53 gene in primary breast tumors, Cancer Res 1991;51:6194-8.

Merlo GR, Venesio T, Bernardi A, Canale L, Gaglia P, Lauro D, Cappa APM, Callahan R, Liscia DS. Loss of heterozygosity on chromosome 17p13 in breast carcinomas identifies tumors with high proliferative index, Am J Path 1992;140:215-23.

Bieche I, Champeme MH, Matifas F, Hacene K, Callahan R, Lidereau R. Loss of heterozygosity on chromosome 7q associated with aggressive primary breast tumors, Lancet 1992;339:139-43.

Callahan R, Cropp CS, Merlo GR, Liscia DS, Cappa APM, Lidereau R. Somatic mutations in human breast cancer: A status report, Cancer 1992;69:1582-8.

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

PROJECT NUMBER
Z01 CB 05148-13 LTIB

PERIOD COVERED
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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,

Robert Callahan	Chief, Oncogenetics Section	LTIB, DCBDC, NCI
Gilbert Smith	Research Biologist	LTIB, DCBDC, NCI
Daniel Gallahan	Senior Staff Fellow	LTIB, DCBDC, NCI
Francesca Diella	Guest Worker	LTIB, DCBDC, NCI
Edith Kordon	Visiting Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Drs. Antoni Marchetti and Francesco Squartini, Univ. of Pisa, Pisa, Italy; Drs. Glenn Merlino and Chameli Jhappen, NCI; Dr. Lothaaar Hennighausen, NIDDK, NIH.

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Oncogenetics Section

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

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	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 study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with the mouse mammary tumor virus (MMTV). MMTV appears to induce tumors by acting as an insertional mutagen that leads to the activation of a previously silent cellular gene or the rearrangement of a normally expressed gene (*int* genes). We have found a dichotomy in the frequency with which the *wnt-1* gene is activated in tumors arising within preneoplastic hyperplastic outgrowth lines (6%) and those arising *in situ* (52%) in the mammary glands of C3H breders. It would appear that *wnt*-1 activation provides a proliferative advantage to transformed mammary epithelial cells in ntact C3H mammary glands. We have determined the nucleotide sequence of the 2.3kb RNA species whose expression is activated by MMTV insertion in the *int*-3 locus in mammary tumors. It encodes a 57kD protein which is 50% identical to the intracellular portion of the neurogenic Drosophilia *notch* gene product. A common characteristic of these proteins is six nearly contiguous 32 amino acid repeats which are bounded by the PEST amino acid sequence motif that is characteristic of proteins having a rapid turnover. We have used the "normal" HC11 mouse mammary epithelial cell line to study the biological activity of the *int*-2 and *int*-3 gene products. *Int*-2 is a member of the fibroblast growth factor (FGF) gene family. Activation of expression of either *int*-2 or *int*-3 in HC11 cells induces anchorage-independent growth in soft agar. Moreover, the autocrine expression of *int*-2 in HC11 cells abrogates their requirement for either epidermal growth factor of bFGF priming prior to inductiion of beta-caesin expression with lactogenic hormones. A transgenic mouse strain has been established containing activated *int*-3 as the transgene. Focal and often multiple poorly differentiated mammary and salivary gland adenocarcinomas occur in 100% of the transgenic mice between 2 and 7 months of age. Significantly, mammary glands were arrested in development and were lactation deficient in all female *int*-3 mice. In other studies, we have developed a novel approach to introducing genes into primary mammary epithelial cells to test their biological activity in mammary fat pads, using a retroviral shuttle vector containing LacZ reporter gene.

Major Findings:

We and others have previously shown that the mouse mammary tumor virus (MMTV) induces preneoplastic hyperplastic lesions within which mammary adenocarcinomas develop. MMTV is known to induce mammary tumors by insertional mutagenesis. The expression of five cellular genes, designated as *wnt-1*, *wnt-3*, *int-2*, *hst*, and *int-3* , have been shown to be activated or altered in mouse mammary tumors by MMTV. *wnt-1* is frequently activated in primary mammary tumors arising in MMTV-infected C3H mice, but previous work indicates that *wnt-1* and *int-2* are rarely affected in hyperplastic alveolar nodules or in mammary tumors developing from these hyperplastic mammary outgrowths (HOGS). We compared the activation of *wnt-1*, *int-2* and *int-3* in tumors from virgin and breeding C3H mice, in primary mammary tumors arising in the same individual C3H breeder and in early passages of C3H mammary-derived HOGs. *wnt-1* and *int-2* activation was 6.0% and 0% respectively in primary HOG-derived C3H tumors compared to 52.0% and 14.0% in primary tumors developing *in situ* in C3H breeders. *Int-3* rearrangements were not detected in any of the tumors tested. No difference was found in the frequency of *wnt-1* activation when MMTV-induced tumors from breeding and virgin C3H were compared, suggesting that hormonal status of the host was not a factor. In addition tumor latency in individual C3H breeders did not correlate with *wnt-1* activation and *wnt-1* RNA was not detected in early transplant generations of C3H HOGs suggesting that the absence of *wnt-1* activation in HOG-derived tumors was not due to selection against *wnt-1* expression during transplant growth. At present we cannot explain the dichotomy in *wnt-1* activation frequency in C3H HOGs and their derivative tumors (6%) and mammary tumors arising *in situ* within the glands of C3H breeders (52%) however it would appear that *wnt-1* activation provides some sort of proliferative advantage to transformed mammary epithelial cells in intact C3H mammary glands.

In previous studies we have identified the *int-3* locus in MMTV induced mouse mammary tumors in which the expression of a novel 2.3Kb RNA species is activated as consequence of MMTV insertional mutagenesis. The nucleotide sequence and biological activity of the mammary tumor specific *int-3* RNA species has been determined. It contains an open reading frame which encodes a 57-kD protein. The translated protein possesses six nearly contiguous 32-amino-acid repeats which are related to a similar motif in the *Saccharomyces cerevisiae cdc-10* -encoded cell cycle protein. In addition, the *int-3 cdc-10* repeats are bounded by the PEST amino acid sequence motif which is commonly found in proteins having a rapid turnover and may represent sites for phosphorylation. The *int-3 cdc-10* repeat sequences are 50% identical to a portion of the intracellular domain of the neurogenic Drosophila *notch* gene product.

We have used the HC11 mouse mammary epithelial cell line study in vitro the biological activity of *int-2* and *int-3* gene products. HC11 cells are not transformed and have retained the ability to differentiation and synthesize the protein beta-casein in response to hormonal stimulation. Activation of expression of a recombinant *int-3* genomic DNA fragment encoding the 2.3-kb RNA species in HC11 cells induces anchorage-independent growth in soft agar. The *int-2* gene product is related to the basic fibroblast growth factor (bFGF). We have shown that in HC11 cells infected with *int-2* retroviral expression vectors, the *int-2* protein can function as a bFGF-like growth factor in stimulating: (a) HC11 cell proliferation in monolayer, and (b) anchorage independent growth in soft agar. The autocrine expression of *int-2* in HC11 cells abrogates their requirement for either exogenous epidermal growth factor or bFGF priming prior to induction of beta-caesin expression with lactogenic hormones.

To determine the *in vivo* consequences of activated *int-3* expression, transgenic mice were generated harboring the same genomic DNA fragment used in the experiments with the HC11 cells. All six founder transgenic mice and the progeny of one established line exhibited similar dramatic phenotypic abnormalities in tissues in which the transgene was expressed. Focal and often multiple poorly differentiated mammary and salivary adenocarcinomas appeared in the majority of transgenic mice between 2 and 7 months of age. Significantly, mammary glands were arrested in development and were lactation deficient in all female *int-3* mice. These findings demonstrate *in vivo* that expression of the activated notch-related *int-3* gene causes deregulation of normal developmental controls and hyperproliferation of glandular epithelia.

The whey acidic protein (WAP) gene is expressed in mammary epithelial cells at late pregnancy and throughout lactation. In collaboration with Dr. Lothar Hennighausen, we have studied transgenic mice in which WAP is expressed from the transgene precociously in pregnancy. From 13 founder mice bearing WAP transgenes, two females and the daughters from one male founder failed to lactate and nurture their offspring. We named this phenotype *milchlos*. Mammary tissue from postpartum *milchlos* mice was underdeveloped, contained too few alveoli and resembled the glands of non-transgenic mid-pregnant mice. The hypothesis that alveolar development in *milchlos* mice was functionally arrested in a prelactational state is consistent with low levels of alpha lactalbumin mRNA and an unidentified keratin RNA in mammary tissue from postpartum mice. Defects in alveolar function in *milchlos* mice were detected at mid-pregnancy; in non-transgenic mice, WAP was secreted into the alveolar lumen but remained preferentially in the cytoplasm of the alveolar epithelial cells in the *milchlos* mice. Since deregulated WAP expression resulted in impaired mammary development, it is possible that WAP plays a regulatory role in the terminal differentiation and development of mammary alveolar cells.

Transgenic technology in which engineered DNA is stably introduced into the mammalian germ line provides whole organisms which provide novel insights into the molecular basis of developmental and pathological processes. The mouse mammary gland provides an excellent experimental model within which the actions of transgenes can be evaluated within a growing, developing and functioning organ system in vivo. Non-immortalized mouse mammary epithelial cells expressing *E. coli* beta-galactosdase from a murine amphotropic packaged retroviral vector were injected into epithelium-divested mammary fat pads of syngeneic mice. Mammary glands formed from the injected mammary epithelial cells contained ductal and lobular cells, both of which expressed beta-galactosidase when examined in situ more than 12 months later. These results indicate that stable recombinant gene expression can be achieved in vivo in the mammary gland without altering the functional or growth properties of normal mammary epithelium.

Publications:

Burdon T, Wall RJ, Shamay A, Smith GH, Hennighausen L. Over expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype, Mech of Development 1991;36:67-74.

Smith GH, Gallahan D, Zwiebel JA, Freeman SM, Bassin RH, Callahan R. Long term *in vivo* expression of genes introduced by retroviral-mediated transfer into mammary epithelial cells, J Virol 1991;65:6365-70.

Venesio T, Taverna D, Hynes NE, Deed R, MacAllen D, Ciardiello F, Valverius EM, Salomon DS, Callahan R, Merlo G. The *int-2* gene product acts as a growth factor and substitutes for bFGF in promoting the differentiation of a normal mouse mammary epithelial cell line, Cell Growth & Diff 1991;3:63-71.

Robbins J, Blondel BJ, Gallahan D, Callahan R. Mouse Mammary tumor gene *int-3*: A member of the Notch gene family transforms mammary epithelial cells, J Virol 1992;66:2594-9.

Jhappan C, Gallahan D, Stahle C, Chu E, Smith GH, Merlino GT, Callahan R. MMTV-activated *int-3* induces mammary and salivary neoplasia and ductular epithelial hyperplasia in transgenic mice, Genes & Dev 1992;6:345-55.

Schwartz MS, Smith GH, Medina D. The effect of parity, tumor latency and transplantation on the activation of *int* loci in MMTV-induced, transplanted C3H mammary preneoplasias and their tumors, Int J Cancer 1991; in press.

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

PROJECT NUMBER
Z01 09023-06 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cloning of Anti-Tumor Antigen Immunoglobulin Genes and Modified Constructs

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

Syed Kashmiri	Expert	LTIB, DCBDC, NCI
Liming Shu	Visiting Associate	LTIB, DCBDC, NCI
William Shupert	Visiting Fellow	LTIB, DCBDC, NCI
Jaymie Sawyer	Guest Researcher	LTIB, DCBDC, NCI
Lucy DeMario	BPT	LTIB, DCBDC, NCI
Hyun-Sil Lee	Visiting Fellow	LTIB, DCBDC, NCI
Patricia Horan Hand	Chemist	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
E. Padlan, Laboratory of Molecular Biology, NIDDK, NIH; M. Whitlow, Enzon; D. Johnson, Eli Lilly.

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
8.1	6.1	2.0

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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 main objective of this research project is to genetically engineer immunoglobulin genes to study structure-function relationships and to generate potentially useful immunological reagents for diagnosis and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) with selective reactivity to various tumor types. These MAbs include those that recognize carcinoembryonic antigen, and a high molecular weight, mucin-like pancarcinoma antigen, TAG-72. The MAbs are currently being evaluated in a number of diagnostic and therapeutic trials on breast, colon and ovarian cancers. The murine MAbs, B72.3 and its second generation counterpart MAb CC49, which recognize TAG-72, have shown promise for being developed into diagnostic and therapeutic agents. A major impediment to the clinical application of murine MAbs is their potential to elicit human anti-mouse antibody (HAMA) response in some patients. This problem can be effectively minimized by genetically replacing the constant region of the mouse antibody with the constant region of human antibody as well as with other genetically engineered manipulations.

Major Findings

The main objective of this research project is to genetically engineer immunoglobulin genes to generate potentially useful immunological reagents for diagnosis and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) with selective reactivity to various tumor types. These MAbs include those that recognize carcinoembryonic antigen or a high molecular weight, mucin-like pancarcinoma antigen, TAG-72. The MAbs are currently being evaluated in a number of diagnostic and therapeutic trials on breast, colon and ovarian cancers. The murine MAb, B72.3 and its second generation counterpart MAb CC49, which recognize TAG-72, have shown promise for being developed into diagnostic and therapeutic agents. A major impediment to the clinical application of murine MAbs is their potential to elicit human anti-mouse antibody (HAMA) response in some patients. This problem can be effectively minimized by genetically replacing the constant region of the mouse antibody with the constant region of human antibody.

With the aim of minimizing HAMA response in patients, we generated recombinant chimeric B72.3, designated cB72.3(γ1), containing the murine variable regions V_H and V_L, and the human kappa and γ1 constant regions. To generate cB72.3(γ1), we cloned the cDNA copies of the messages as well as the rearranged genomic sequences encoding the heavy and the light chains of MAb B72.3. Construction of mouse-human chimeric heavy and light chain genes was performed by inserting DNA fragments encoding V_L and V_H regions of B72.3 into unique restriction sites of expression vector carrying sequences encoding constant regions of a human kappa and γ1, respectively. The kappa and γ1 expression constructs were sequentially introduced into SP2/0 cells by electroporation. A stable transfectoma producing 10-20 μg/ml of cB72.3(γ1) was developed and the cB72.3(γ1) was characterized. Reciprocal competition radioimmunoassays demonstrated that cB72.3(γ1), cB72.3(γ4) and native B72.3 (designated nB72.3) competed similarly. Also, cB72.3(γ1) was indistinguishable from the nB72.3 in its binding to a rat anti-idiotype made against nB72.3. Characterization of cB72.3(γ1) showed a higher isoelectric point for cB72.3(γ1) compared to nB72.3 and cB72.3(γ4). SDS-PAGE analysis revealed slight differences in size among the three MAb forms. cB72.3(γ1) mediated better tumor cell killing than either nB72.3 or cB72.3(γ4). Dual label studies of coinjected cB72.3(γ1) and nB72.3 revealed that both MAbs could efficiently localize human tumor xenografts in athymic mice. Pharmacokinetic studies, analyzing the blood clearance of cB72.3(γ1), cB72.3(γ4) and nB72.3 in mice, showed that β phase of the nB72.3 clearance was slower than that of the other MAb forms. However, when the pharmacokinetic patterns of these three MAb forms were analyzed in monkeys, the cB72.3(γ1) and the nB72.3 showed similar clearance curves, while the cB72.3(γ4) showed a much slower plasma clearance. In view of the binding properties of nB72.3 and its ability to localize a range of carcinomas in clinical trials, the studies reported here demonstrate that the cB72.3(γ1) may serve as a potentially useful diagnostic and/or therapeutic reagent.

The constant region domains of the immunoglobulin chain determine antibody effector functions, such as complement fixation and antibody dependent cellular cytotoxicity activity. These domains also determine the serum half-life and the degree of *in vivo* localization of the antibody. Genetically engineered deletions of various heavy chain domains of an antibody could maximize the desired while reducing or eliminating the unwanted effector functions. These alterations, in turn, could generate immunological reagents with an optimal clearance rate and desired in vivo localization. More recently, we have attempted a variety of genetic manipulation to generate such desired immunological reagents with an optimal rate of antibody clearance from the serum using site-directed mutagenesis. We are in the process of engineering cDNA clones of MAb B72.3 that may encode aglycosylated and domain-deleted cB72.3(γ1).

In our efforts to optimize pharmacokinetics of plasma clearance and efficacy of tumor localization and penetrance of the anti-tumor antibodies, we have developed and characterized a novel type of recombinant molecule, a single-chain antigen binding protein. The molecule, also termed a single chain Fv(sFv) is composed of the variable domain of the light chain tethered to the variable domain of the heavy chain via a flexible linker peptide. To develop a single chain Fv of MAb CC49, cDNA copies of the messages encoding the heavy and light chains of MAb CC49 were cloned in the phage vector following subcloning of the restriction fragments encoding V_H and V_L regions in separate M13 vectors. Primer-induced site directed mutagenesis was used to generate appropriate restriction endonuclease sites and stop codons in the V_L and V_H sequences. A synthetic oligonucleotide carrying appropriate restriction site flanks and encoding the peptide linker, the last four residues of V_L and the first three residues of V_H was first ligated to the DNA fragments encoding V_H segments. The resulting DNA molecule, the V_L fragment and the linearized prokaryotic expression vector carrying appropriate restriction ends were assembled together in a 3 way ligation. The completed sFv expression construct was introduced into E. coli expression host. The sFv protein was expressed, extracted, renatured, and purified.

The sFv was shown to be a homogenous protein of approximately M_r 27,000 which could be efficiently radiolabeled with ^{125}I or ^{131}I. Competition radioimmunoassays revealed that relative binding affinity of sFv, like that of CC49 Fab', was 8-fold lower than the intact CC49 IgG and the dimeric $F(ab')_2$. Nonetheless, the ^{131}I-labeled sFv was shown to bind biopsies of TAG-72 expressing tumors. CC49 sFv showed extremely rapid whole body clearance in mice and a very rapid plasma clearance in both mice and monkeys. Tumor targeting studies with all four radiolabeled Ig CC49 forms, using the LS-174T human colon carcinoma xenograft model, revealed a much lower percentage injected dose/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 (radiolocalization indices) for the sFv were comparable to or greater that 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. Gamma scanning studies also showed that ^{131}I-CC49 sFv could efficiently localize tumors. The CC49 sFv may thus have utility in diagnostic and perhaps therapeutic applications for a range of human carcinomas.

Publications

Hutzell P, Kashmiri S, Colcher D, Primus FJ, Horan Hand P, Roselli M, Finch MD, Yarranton G, Bodmer M, Whittle N, King D, Loullis CC, McCoy DW, Callahan R, Schlom J. Generation and characterization of a recombinant/chimeric B72.3 (human γ1), Cancer Res 1991;51:181-9.

Milenic DE, Yokota T, Filpula DR, Finkelman MAJ, Dodd SW, Wood JF, Whitlow M, Snoy P, Schlom J. Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49, Cancer Res 1991;51:6363-71.

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

PROJECT NUMBER
Z01 CB 05216-21 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Site-Selective cAMP Analogs as Antineoplastics and Chemopreventives

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

Yoon S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBDC, NCI
Timothy Clair	Chemist	LTIB, DCBDC, NCI
Alfredo Budillon	Visiting Fellow	LTIB, DCBDC, NCI
Anna Cereseto	Visiting Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. B. Jastorff, University Bremen, FRG; DR. W.R. Miller, U. of Edinburgh, Scotland; Dr. A. Pinto, Centro di Riferimento Oncologico, INRCCS, Aviano, Italy; Dr. G. Tortora, U. of Naples Medical School II, Naples, Italy; Dr. G. Weber, Indiana School of Medicine, Indianapolis, IN.

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SECTION
Cellular Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Betheda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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☐ (a1) Minors
☐ (a2) Interviews
B

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

Cyclic AMP (cAMP) in mammalian cells functions by binding to cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase. The cAMP receptor protein has two different cAMP binding sites, and cAMP analogs that selectively bind to either one of the two binding sites are known as site A-selective (C-6 analogs) and site B-selective (C-2 and C-8 analogs). We discovered that site-selective cAMP analogs exhibit potent growth inhibition *in vitro* and *in vivo* in a range of human carcinomas, fibrosarcomas, and leukemias without causing cytotoxicity. Site-selective cAMP analogs are also promising in view of their ability to act synergistically as non-toxic differentiation agents at low, micromolar doses, not only with each other, but also in combination with other differentiating agents currently accepted for clinical use. 8-Cl-cAMP, the most potent site-selective cAMP analog, also exerts potent growth inhibition of both p-glycoprotein (pgp)-associated and -unassociated multidrug resistant human cancer cell lines. The molecular mechanism for such potency in the growth inhibitory effect of 8-Cl-cAMP and other site-selective cAMP analogs takes advantage of the ability of these analogs to selectively modulate two isoforms of cAMP receptor proteins, type I and type II protein kinase, the positive and negative regulators of cell growth and differentiation. 8-Cl-cAMP markedly down-regulate the growth stimulatory protein, type I protein kinase while upregulating the growth inhibitory protein type II protein kinase. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancer.

Major Findings:

Effects of 8-chloroadenosine 3', 5'-monophosphate and N6-benzyl-cyclic adenosine 3', 5'-monophosphate on cell cycle kinetics of HL-60 leukemia cells. Site-selective cyclic AMP (cAMP) analogues have been shown to inhibit growth and induce differentiation in several human leukemia cell lines. However, detailed studies of the effects exerted by cAMP analogues on cell cycle kinetics have been lacking. We have examined the effects of 8-Cl-cAMP and N6-benzyl-cAMP on the cell cycle kinetics of the HL-60 human promyelocytic leukemia cell line. A cell cycle study was performed by univariate DNA analysis after 24-72 h of treatment with noncytotoxic concentrations of 8-Cl-cAMP and N6-benzyl-cAMP capable of inducing 50-60% growth inhibition in these cells. HL-60 cells treated with 5 μM 8-Cl-cAMP showed no significant change in the cell distribution in the cycle as compared to the untreated control cells, whereas the treatment with 10 μM M6-benzyl-cAMP transiently increased the percentage of cells in the G0/G1 phase after 48 h, followed by a partial recovery at 72 h. Combined treatment with low doses of 8-Cl-cAMP and N6-benzyl-cAMP, each of which alone produced 20% growth inhibition, exerted a growth inhibitory effect of 65% and delayed increase of the G0/G1 phase by 72 h. To better understand the cell cycle effects induced by 8-Cl-cAMP, flow cytometric analysis of bromodeoxyuridine incorporation was also performed. 8-Cl-cAMP treatment exhibited a slowing down of the cell cycle; thus, the delayed appearance of the G0/G1 cell accumulation after combined treatment could be due to this effect of 8-Cl-cAMP on the HL-60 cell cycle. At a toxic dose, 8-Cl-cAMP brought about a G2M block, whereas N6-benzyl-cAMP brought about an increase of the G0/G1 compartment. G2M block produced by toxic doses of 8-Cl-cAMP was not related to its adenosine metabolite since 8-Cl-adenosine did not produce any specific block in the cell cycle. Our results show, for the first time, that these site-selective cAMP analogues could affect cell cycle kinetics at different points. These data may provide the basis for combination treatments involving cAMP analogues and other agents in the treatment of human leukemia.

Tiazofurin and 8-Cl-cAMP action in human ovarian and pancreatic carcinoma cells. Tiazofurin and 8-Cl-cAMP are novel chemotherapeutic agents shown to be effective against various cancer cells *in vitro* and *in vivo*. They act through distinct mechanisms, modulating the signal transduction pathway, causing growth inhibition, differentiation and down-regulation of c-ras and c-myc oncogene expression. We examined the action of tiazofurin and 8-Cl-cAMP on colony formation in HT-29 human colon carcinoma and BxPC-3 and PANC-1 human pancreatic carcinoma cell lines. The IC50 of 8-Cl-cAMP was 0.1 and 0.2 μM in the pancreatic and colon cancer cell lines, respectively, and tiazofurin yielded IC50s from 4(PANC-1) to 18 (HT-29) μM. Simultaneous incubation with tiazofurin and 8-Cl-cAMP provided additive effects on cytotoxicity, measured by clonogenic assay, in all 3 cell lines. The results indicate the possible clinical usefulness of a combination of tiazofurin and 8-Cl-cAMP in the treatment of colon and pancreatic carcinoma.

Unhydrolyzable analogues of adenosine 3':5'-monophosphate demonstrating growth inhibition and differentiation in human cancer cells. A set of adenosine 3':5'-monophosphate (cAMP) analogues that combine exocyclic sulfur substitutions in the equatorial (Rp) or the axial (Sp) position of the cyclophosphate ring with modifications in the adenine base of cAMP were tested for their effect on the growth of HL-60 human promyelocytic leukemia cells and LS-174T human colon carcinoma cells. Both diastereomers of the phosphorothioate derivatives were growth inhibitory, exhibiting a concentration inhibiting 50% of cell proliferation of 3-100 μM. Among the analogs tested, Rp-8-Cl-cAMPS and Sp-8-Br-cAMPS were the two most potent. Rp-8-Cl-cAMPS was 5- 10-fold less potent that 8-Cl-cAMP while Sp-8-Br-cAMPS was approximately 6-fold more potent than 8-Br-cAMP. The growth inhibition was not due to a block in a specific phase of the cell cycle or due to cytotoxicity. Rp-8-Cl-cAMPS enhanced its

growth-inhibitory effect when added together with 8-Cl-cAMP and increased differentiation in combination with N6-benzyl-cAMP. The binding kinetics data showed that these Sp ands Rp modifications brought about a greater decrease in affinity for Site B than for Site A of RI (the regulatory subunit of type I cAMP-dependent protein kinase) and a substantial decrease of affinity for Site A of RII (the regulatory subunit of type II protein kinase) but only a small decrease in affinity for Site B of RII, indicating the importance of the Site B binding of RII in the growth -inhibitory effect. These results show that the phosphorothioate analogues of cAMP are useful tools to investigate the mechanism of action of cAMP in growth control and differentiation and may have practical implication in the suppression of malignancy.

Publications

Cho-Chung YS, Yokozaki H, Tortora G, Pepe S, Clair T. 8-Cl-cAMP in differentiation therapy: The reversal and suppression of malignancy targeting the intracellular transducing proteins of cAMP. In: Waxman S, Rossi GB, Takaku F, eds. The Status of Differentiation Therapy of Cancer. Vol II. New York: Raven Press, 1991;185-98.

Cho-Chung YS, Torotra G, Pepe S, Yokozaki H. The differentiation of HL-60 human promyelocytic leukemia by 8-Cl-cAMP and rhGM-CSF in combination. In: Waxman S, Rossi GB, Takaku F, eds. The Status of Differentiation Therapy of Cancer. Vol II. New York: Raven Press, 1991;393-98.

Cho-Chung YS, Clair T, Tortora G, Yokozaki H. Review: Role of site-selective cAMP analogues in the control and reversal of malignancy, Pharmacology and Therapeutics 1991;50:1-33.

Cho-Chung YS. Suppression of malignancy targeting transducing proteins of cyclic AMP signal. In: 91 Summer Symposium of Korean Federation of Science and Technology Societies. Seoul:KOFST Press 1991;137-40.

Cho-Chung YS. Correspondence re: M.M. Van Lookeren Campane, et al. 8-chloroadenosine 3', 5'-monophosphate inhibits the growth of Chinese hamster ovary and Molt-4 cells through its adenosine metabolite, Cancer Res 1991;51:6206-8.

Guadagni F, Tortora G, Clair T, Cho-Chung YS, Schlom J, Greiner JW. Carcinoembryonic antigen regulation in human colorectal tumor cells by a site-selective cyclic AMP analogue - a comparison with interferon-gamma, Int J Cancer 1991;48:413-22.

Pepe S, Tortora G, Noguchi PD, Marti GE, Washington GC, Cho-Chung YS. Effects of 8-Cl-cAMP and N6-Benzyl-cAMP on cell cycle kinetics of HL-60 leukemia cells, Cancer Res 1991;51:6263-7.

Yokozaki H, Tortora G, Pepe S, Maronde E, Genieser HG, Jastorff B, Cho-Chung YS. Unhydrolyzable analogs of adenosine 3',5'-monophosphate demonstrating growth inhibition and differentiation in human cancer cells, Cancer Res 1992;52:2504-8.

Cho-Chung YS. Suppression of malignancy targeting cyclic AMP signal transducing proteins. In: Biochemical Society Transactions. London: Portland Press, 1992;20:425-30.

Cho-Chung YS. Modulation of adenylate cyclase signalling. In: Workman P, ed. Seminars in Cancer Biology: New Anticancer Drug Desgign and Discovery Based on Advances in Molecular Oncology. London: Saunders Scientific Publications, 1992; in press.

Szekeres T, Cho-Chung YS, Weber G. Action of tiazofurin and 8-Cl-cAMP in human colon and pancreatic cancer cells, Cancer Biochem Biophys 1992; in press.

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

PROJECT NUMBER
Z01 CB 08281-10 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mechanism of cAMP Action in Growth Control, Differentiation, and Gene Regulation

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

Yoon-S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBDC, NCI
Alfredo Budillon	Visiting Fellow	LTIB, DCBDC, NCI
Timothy Clair	Chemist	LTIB, DCBDC, NCI
Christian Rohlff	Special Volunteer	LTIB, DCBDC, NCI
Anna Cereseto	Visiting Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. Tore Jahnsen, Institute of Pathology, Rikshospitalet, Oslo, Norway; Dr. R. Tahara, Hiroshima Univ. Med. School, Hiroshima, Japan; Dr. D. Ogreid, U. of Bergen, Norway; and Dr. E.S. Severin, Res. Center of Molecular Diagnostics and Therapy, Moscow.

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NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
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☐ (a2) Interviews
B

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

In normal tissues, the balance between the positive and negative regulation with respect to cell proliferation is precisely controlled at the level of the cell surface, which receives extra-cellular signals, and at the intracellular level where these signals are transduced. Alterations or breakdown of these growth regulatory circuits, the growth stimulatory and growth-constraining mechanisms, are involved in triggering the process of uncontrolled outgrowth: cancer.

Our hypothesis is that cAMP-dependent protein kinases are crucial effectors in tumorigenesis. cAMP acts by binding to the regulatory subunits of cAMP-dependent protein kinase. Two such subunits exist, RI and RII, which interact with a common catalytic subunit and are present in normal cells as a specific physiological ratio; departure from the normal balance of these two isoforms of the subunits may lead to the induction of malignant transformation. cAMP binds to RI and RII; however, these cAMP receptor proteins transduce opposite signals, the RI being stimulatory and the RII inhibitory of cell proliferation. This conclusion was drawn from the studies that employed independent experimental approaches: the use of site-selective cAMP analogs that, unlike parent cAMP, are able to differentiate between the binding sites on RI and RII; antisense oligonucleotides, those that are able to selectively inhibit the function of RI and RII; and transfer and overexpression of RI or RII gene by a retroviral vector.

These studies demonstrated that restoration of the normal balance between RI and RII is of great potential in cancer therapy. Thus, these studies contribute to understanding the mechanism of cAMP control of cell growth and differentiation and provide new approaches to the treatment of cancer.

Major Findings

Formation of a truncated regulatory subunit correlates the suppression of type I cAMP-dependent protein kinase (PKA-I) in the 8-Cl-cAMP induced differentiation of HL-60 leukemia cells. 8-Cl-cAMP induces growth inhibition and differentiation in a broad spectrum of human cancer cell lines. Such effects correlate with the ability of 8-Cl-cAMP to suppress PKA-I and enhance PKA-II expression. We have investigated the levels of PKA-I and -II in HL-60 leukemia cells following 8-Cl-cAMP treatment using DEAE-cellulose - high performance liquid chromatography. In the cytosols of untreated cells, two major peaks (peaks 1 & 2) of PKA activity that were coincident with the peaks of cAMP binding activity were found. Peaks 1 & 2 were eluted at 70 and 180 mM NaCl, respectively, and the kinase and binding activities of peak 1 were ~ 4-fold that of peak 2. Photoaffinity labeling of the eluents with 8-N3-[^{32}P]cAMP showed that peak 1 contained RIa (48 KDa), while peak 2 contained RIIb (52KDa). When cells were treated for 3 days with 8-Cl-cAMP, peak 1 decreased to 30% of those in the untreated cells, while peak 2 increase 2~3-fold over that of untreated cells. In addition, the photoaffinity labeling detected a new species of RI (34KDa) in peak 1. These results were confirmed when cells were treated with 8-Cl-[^{3}H]-cAMP. Thus, a truncated RIa subunit retaining the binding ability to both C subunit and cAMP is involved in the suppression of PKA-I expression by 8-Cl-cAMP.

Chemical modification enhances the inhibitory effect of regualtory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase type I on cell proliferation. The analysis of the action of antisense oligodeoxynucleotide complementary to mRNA of the type I cAMP-dependent protein kinas regulatory subunit has revealed a stable inhibitory effect of the substance on Molt-4 leukemic cell proliferation. To enhance the efficiency of the oligodeoxynucleotide action, its molecule was subjected to terminal modification. The hydrophobic radical-effected modification at the 5'-end was carried out at the last stage of automated synthesis using undecanol. At the 3'-end, the molecule was modified with an acridine residue. The substances synthesized were significantly more potent in inhibiting thymidine incorporation in the acid insoluble fraction, compared to the nonmodified nucleotide. Examination of the cell cycle progression of Molt-4 cells has demonstrated a considerable shift of cells from G_2/M stage to G_1, the process being more effective in the case of modified oligonucleotides.

8-Cl-cAMP acts synergistically with tumor necrosis factor (TNF), TGFβ, or retinoic acid (RA) in growth inhibition and differentiation of HL-60 human promyelocytic leukemia cells. HL-60 cells respond to various signals by differentiating to more mature cells. 8-Cl-cAMP, TGFβ, or TNF each differentiates these cells to monocyte/ macrophages, whereas RA differentiated these cells to granulocytes. By the use of antisense strategy, it has been shown that type II regulatory subunit (RIIβ) of cAMP-dependent protein kinase is essential in the cAMP-induced differentiation of HL-60 cells. The present study examined effects of combination of 8-Cl-cAMP with TNF, TGFβ, or RA on the growth and differentiation of HL-60 cells. When 8-Cl-cAMP was added to these cells in combination with either TNF, TGFβ, or RA at the concentrations at which each alone produces 10-15% growth inhibition, a synergistic effect was observed: growth inhibition was enhanced to 50-70% with synergism coefficients of 2.3, 1.6, and 2.1, respectively. This synergism on the growth inhibition accompanied the induction of differentiation markers and change in cell morphology. The mechanism of the synergistic action

of these differentiating agents is being studied by the use of antisense strategy and retroviral vector-mediated gene transfer technology.

Publications

Cho-Chung YS, Clair T, Tortora G, Yokozaki H. Review: Role of site-selective cAMP analogues in the control and reversal of malignancy, Pharmacology and Therapeutics 1991;50:1-33.

Tortora G, Pepe S, Yokozaki H, Meissner S, Cho-Chung YS. Cooperative effect of 8-Cl-cAMP and rhGM-CSF on the differentiation of HL-60 human leukemia cells, Biochem Biophys Res Commun 1991;177:1133-40.

Cho-Chung YS, Yokozaki H, Tortora G, Pepe S, Clair T. 8-Cl-cAMP in differentiation therapy: The reversal and suppression of malignancy targeting the intracellular transducing proteins of cAMP. In: Waxman S, Rossi GB, Takaku F, eds. The Status of Differentiation Therapy of Cancer. Vol II. New York: Raven Press, 1991;185-98.

Cho-Chung YS, Torotra G, Pepe S, Yokozaki H. The differentiation of HL-60 human promyelocytic leukemia by 8-Cl-cAMP and rhGM-CSF in combination. In: Waxman S, Rossi GB, Takaku F, eds. The Status of Differentiation Therapy of Cancer. Vol II. New York: Raven Press, 1991;393-98.

Cho-Chung YS. Suppression of malignancy targeting transducing proteins of cyclic AMP signal. In: 91 Summer Symposium of Korean Federation of Science and Technology Societies. Seoul:KOFST Press 1991;137-40.

Cho-Chung YS. Correspondence re: M.M. Van Lookeren Campane, et al. 8-chloroadenosine 3', 5'-monophosphate inhibits the growth of Chinese hamster ovary and Molt-4 cells through its adenosine metabolite, Cancer Res 1991;51:6206-8.

Yokozaki H, Tortora G, Pepe S, Maronde E, Genieser HG, Jastorff B, Cho-Chung YS. Unhydrolyzable analogs of adenosine 3',5'-monophosphate demonstrating growth inhibition and differentiation in human cancer cells, Cancer Res 1992;52:2504-8.

Cho-Chung YS. Suppression of malignancy targeting cyclic AMP signal transducing proteins. In: Biochemical Society Transactions. London: Portland Press, 1992;20:425-30.

Cho-Chung YS. Modulation of adenylate cyclase signalling. In: Workman P, ed. Seminars in Cancer Biology: New Anticancer Drug Desgign and Discovery Based on Advances in Molecular Oncology. London: Saunders Scientific Publications, 1992; in press.

Cho-Chung YS, Nesterova MV, Severin ES, Vinogradov SV. Chemical modification enhances the inhibitory effect of regulatory subunit antisense oligonucleotide of cAMP-dependent protein kinase type I on cell proliferation, Biochem Intl 1991;25:767-73.

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

PROJECT NUMBER
Z01 CB 04848-20 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
"Anti-Oncogenes": The Analysis of Cellular Resistance to Transformation

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

Mary Lou McGeady Cutler	Expert	LTIB, DCBDC, NCI
Lorenzo Zanoni	Guest Worker	LTIB, DCBDC, NCI
Toshitaka Tsuda	Fogarty Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. Makoto Noda, Riken Institute of Science, Japan

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Experimental Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0

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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 have constructed a cDNA library from a *ras* revertant cell line, in a eukaryotic expression vector and screened this library for cDNAs which are 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. Two cDNAs isolated using this strategy have been found on secondary screening to be capable of suppressing the *ras* transformed phenotype. The first of these cDNAs, referred to as *rsp*-1, is a novel gene which specifically suppresses v-Ki-*ras* and v-Ha-*ras* transformation of fibroblasts and epithelial cells. The *rsp*-1 protein contains a series of leucine based repeats homologous to those found in the putative ras binding region of yeast adenylyl cyclase. These findings in conjunction with the fact that it is a phylogenetically highly conserved protein suggests that *rsp*-1 may physically associate with *ras* p21. In addition, we have identified a small RNA, 4.5S RNA, as 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. Our current efforts are aimed at determining the mechanisms by which these two molecules disrupt v-*ras* signal transduction.

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 the *ras* transformation and to characterize the mechanisms and pathways necessary for their phenotype 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. cDNAs 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 first of these clones is a novel gene referred to as *ras*-1. The introduction of *ras*-1 cDNA into DT cells suppressed the growth of that cell line in agar by 30-75% and yielded phenotypically flat revertants. *ras*-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 NIH3T3 cell line containing a copy of the *rsp*-1 cDNA under the control of a mouse metallothionein promoter was specifically resistant to retransformation by v-Ha-*ras* and v-Ki-*ras* but not by v-*mos*, v-*src* or v-*raf*. Hybridization of a *rsp*-1 specific probe to DNA from a number of eukaryotic species revealed that *rsp*-1 is a highly conserved single copy gene. The *rsp*-1 cDNA is not related to any sequences in the nucleic acid data bases. It encodes a 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 regulation of adenylyl cyclase by ras in Sa. cerevisiae. This homology, in conjunction with the *ras* suppressor activity, suggests that *rsp*-1 may associate with *ras* p21 and that its suppressor activity may be a result of this property. Our current studies are aimed at expanding our knowledge of the role *rsp*-1 plays in the regulation of ras signal transduction.

DNA sequence analysis of the other recovered cDNA with ras suppresor activity revealed that it was a clone of 4.5S small nuclear RNA. We have determined that this RNA is expressed at a reduced level in *ras* transformed cells. In addition, it is present at a 5-10 fold higher level in cells resistant to *ras* infection, *i.e., ras* revertants, than in normal cells. The increase in the 4.5S RNA level in the revertant cell lines was attributable to both transcriptional and post-transcriptional events while the reduction in the transformed cells was entirely the result of a decrease in the rate of transcription. Analysis of the level of other small nuclear RNAs in the transformed and revertant cell lines revealed that the mouse B2 repeat sequences share this unusual transcription pattern. Introduction of the gene for 4.5S RNA containing its own promoter elements into a retroviral "double copy" vector allowed us to transfect and infect ras transformed cells thereby increasing the levels of 4.5S RNA in the recipient cells. Using this methodology, it has been possible to suppress the growth of ras transformed cells in soft agar, but no stable revertant cell lines have been isolated.

4.5S RNA was characterized as a RNA molecule found associated with preparations of polyadenylated RNA and a role for this small RNA in transport of mRNA has been proposed. The recent discovery that one of the *ras* GTPase (*ras*-GAP) associated proteins, p62, is an RNA binding protein suggests that regulation of mRNA may be a function of a *ras* signaling pathway. Our current efforts are aimed at determining whether 4.5S RNA is involved in a regulatory pathway with p62 and what the contribution of this small RNA is to maintenance of the nontransformed phenotype.

Publications

Cutler ML, Bassin RH, Zanoni L, Talbot N. Isolation of *rsp*-1, a novel cDNA capable of suppressing v-*ras* transformation, Mol Cell Biol 1992;12.

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

PROJECT NUMBER
ZOI CB 08226-16 LTIB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Hormones and Growth Factors in Development of Mammary Glands and Tumorigenesis

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

Barbara Vonderhaar	Research Chemist	LTIB, DCBDC, NCI
Rina Das	Visiting Associate	LTIB, DCBDC, NCI
Mario Ikeda	Visiting Fellow	LTIB, DCBDC, NCI
Erika Ginsburg	Biologist	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
Dr. Sandra Haslam, Michigan State University, East Lansing, MI; Dr. Claudio Dati, University of Torino, Torino, ITALY, Dr. James Zwiebel, Georgetown University, Washington, DC; Dr. Eva Valverius, Uppsala University Hospital, Uppsala, SWEDEN

LAB/BRANCH
Laboratory of Tumor Immunology and Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 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

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 milleu of the target cell. Our emphasis has been on the interactions of prolactin (Prl), thyroid hormone, and estrogens with recent work also examining how epidermal growth factor (EGF), and EGF-like growth factors are affected by the interplay of these three classical hormones. In addition, we have explored the relationship of a membrane associated antilactogen binding site (ALBS) to the lactogenic hormone receptor on human breast cancer cell growth in culture. 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 α-TGF can promote full lobulo-alveolar development *in vitro*. This effect is not inhibited by β-TGF. The primed mammary gland is more sensitive to α-TGF than to EGF. Prl induced growth of the mouse mammary epithelial cell NOG-8 appears to involve 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. 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 may be intimately associated with the Prl receptor. The antiprolactin action of tamoxifen, working through the ALBS, may have important clinical implications.

Major Findings

In addition to the classical hormones such as insulin (I), corticoids (A and H), prolactin (Prl), estrogen (E) and thyroid hormone (T_3), 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.

Lobulo-alveolar development of the mammary gland it also under the control of growth factors in the epidermal growth factor (EGF) family. Previously we had shown that the *in vitro* induction of lobulo-alveolar development in mouse mammary glands, under the influence of the hormones I, Prl, A and H, also required the presence of EGF in order to occur. We have now shown that a-transforming growth factor (α-TGF) is fully active in promoting lobulo-alveolar development *in vitro*.

Glandular development occurs *in vitro* only after priming with estrogen and progesterone (E/P). This priming increases the ability of the mammary tissue to bind the growth factor as well as induces the production of a mammary derived EGF-like growth factor. This factor is immunologically distinct from EGF but binds to the EGF receptor. It is probably α-TGF as polyclonal antibodies against recombinant α-TGF recognize the factor in extracts from E/P primed mammary glands. Estrogen (E) alone or progesterone (P) alone is not sufficient during the priming process nor can the addition of E and P to the culture medium overcome the need for priming *in vivo*. E/P priming results in a 50% increase in DNA synthesis in the mammary glands. This effect appears to be primarily due to P. Local increases in EGF receptors occur during priming as demonstrated by immunocytochemistry, Western blots and binding with biotin conjugated EGF. Priming with E/P cannot be replaced by EGF priming (either using EGF directly or by elevating salivary gland production of EGF with testosterone). Injecting the animals with anti-EGF during the priming with E/P does not prevent the priming process. All of these data suggest that the role of E and P in priming is a complex one possibly involving positive as well as negative actions by these hormones. Future work will examine the role of each of these hormones in the induction of the growth factor receptors as well as the induction of the EGF-like growth factor (α-TGF) using ovariectomized mice. The characteristics of binding of both EGF and α-TGF to breast tissue will be examined in detail to determine whether the differences in sensitivity to the two related growth factors lies with the receptor or with post-receptor events. In addition, the possibility that the priming removes a natural inhibitor of lobulo-alveolar development (i.e. β-TGF) will be examined.

Under the proper conditions of serum staging, Prl promotes growth of several human breast cancer cell lines including MCF-7, ZR-75.1 and T47D and the "normal" mouse mammary epithelial cell line, NOG-8. Prl-induced growth of NOG-8 cells appears to involve activation of protein kinase C (PKC). Within 5 to 10 min. of addition of Prl to these cells, a 9 - 10 fold increase in PKC activity is observed. This is followed by a rapid decrease in the activity to a level below that of the control within 24hr. The phorbol ester, PMA, also increases PKC activity in these cells but its effects are not additive to those of Prl suggesting a common pathway. Prl treatment causes rapid translocation of PKC from cytosol to membranes. In control cells, 70% of the enzyme activity is in the cytosol. After 5 min. of Prl treatment, 90% of the activity is membrane associated.

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. The order of affinities of the various antiestrogens for the ALBS parallels the order of their potencies as growth and lactogen binding inhibitors. They do not affect the binding of other ligands

to their membrane associated receptors. Both the Prl receptor and the ALBS are primarily localized to the microsomal membranes of the normal mammary cells. Maximal inhibition of Prl binding by TAM is observed in the light microsomes which contain plasma membranes. In addition to the inhibition of Prl binding, TAM also prevents the Prl-induced accumulation of caseins by cultured mouse mammary explants. Future work will characterize the Prl receptor isolated by affinity chromatography and immunopurification and determine what, if any, physical relationship exists between this receptor and the ALBS.

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-prolactin receptor antibody in inhibiting the binding of prolactin to membranes from human placental chorion and human breast tumors. Binding of Prl and other lactogenic hormones to T47D human breast cancer cells *in vitro* was blocked by the addition of B6.2 to the reaction. A non-specific monoclonal antibody, MOPC-2 1, and the anti-NCA monoclonal antibody B1.1, had no effect on binding. Epidermal growth factor receptors on these same cells were unaffected by B6.2. Prl induced growth of the T47D cells was blocked by addition of B6.2 to the media while both B1.1 and MOPC-2 1 were without effect. Specific binding of B6.2 to the cells was completely inhibited by prolactin. Binding of both Prl and B6.2 was inhibited by growing the T47D cells in the presence of tunicamycin A_1 under conditions where protein synthesis was not affected but glycosylation of proteins was. Addition of α-interferon to the culture medium for 72 hr resulted in a 2.5-fold increase in specific B6.2 binding and a 12.5-fold increase in specific lactogenic hormone binding to the live cells. By Western blot analysis only a 2-fold increase in protein was observed suggesting that the interferon was unmasking cryptic prolactin receptors on the cell membranes. An affinity column of B6.2 was used to purify its antigen from T47D cells. The primary purification product, a M_r 90,000 protein, specifically bound the lactogenic hormones human prolactin, human growth hormone, ovine prolactin but not the somatogenic hormone, bovine growth hormone. The purified B6.2 antigen was precipitated by the polyclonal anti-prolactin receptor antibody but not by MOPC-2 1, B1.1 or anti-CEA monoclonal antibodies. When tryptic and V8 digests of the B6.2 antigen and purified prolactin receptors were compared, identical electrophoretic profiles were obtained. Thus these data suggest that the monoclonal antibody B6.2 is an anti-human prolactin 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 prolactin receptor, reacted with B6.2 and polyclonal anti-prolactin receptor antibody, but not with B1.1 or MOPC-21. Parental 3T3 cells, devoid of prolactin receptors, were negative for all antibodies tested. Thus, MAb B6.2, an anti-prolactin receptor antibody, provides a useful tool for further studies on purification and characterization of these receptors from human tissues.

Publications

Ginsburg E, Vonderhaar BK. Stimulation of growth of human breast cancer cells (T47D) by platelet derived growth factor, Cancer Letters 1991;58:137-44.

Vonderhaar BK, Banerjee R. Is tamoxifen also an antilactogen? Mol Cell Endocrinol 1991;79:C159-63.

Vonderhaar BK, Plaut K. Interdependence of hormones and growth factors in lobulo-alveolar development of the mammary gland and in tumorigenesis. In: Dogliotti L, Sapino A, and Bussolati G, eds. Biological and Clinical Progress in Breast Cancer. Boston: Kluwer Academic Publishers, 1992;59-80.

Vonderhaar BK, Plaut K. Mammary development and morphogenesis. In: Donnelly P and McDowell GH, eds. Molecular Biology and Milk Production. Melbourne, Australia: DRDC Press, 1991;1-23.

Vonderhaar BK, Banerjee R, Ginsburg E, Bhattacharjee M, Biswas R. Prolactin signal transduction and steroid requirements in controlling mammary cells. In: Donnelly P and McDowell GH, eds. Molecular Biology and Milk Production. Melbourne, Australia: DRDC Press, 1991;86-97.

Vonderhaar BK, Plaut K, Freeman S, Zweibel J. A transgenic mammary gland in a normal animal. In: Donnelly P and McDowell GH, eds. Molecular Biology and Milk Production. Melbourne, Australia: DRDC Press, 1991;148-162.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08907-09 OD

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT *(80 charactars 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, laboratory, and institute affiliation)*

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)*

Dr. Shu-Mei Liang and Dr. Chi-Ming Liang from the Center for Biologics Evaluation and Research, FDA

LAB/BRANCH

Office of the Director, DCBDC

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

1. Protein kinase C and cytokine regulation of CD3-AK response induced by αCD3. Induction of CD3-AK killer cells is predominantly a PKC-dependent event; however, there is a PKC-independent pathway of CD3-AK cell generation regulated by IL-4. In the PKC-dependent pathway, PKC regulates the production of cytolytic granules but does not directly affect the lytic activity of these granules. In the PKC-independent pathway, IL-4 is the major lymphokine which regulates the production of cytolytic granules. IL-2 regulates both the cytolytic activity and the growth of CD3-AK cells in the PKC-dependent pathway of CD3-AK response. In the PKC-independent pathway, the major role of IL-2 is to support the growth of CD3-AK cells.

2. Differential regulation by cytokines and PKC of the proliferative and cytotoxic responses to αCD3 activation of resting lymphocytes and preactivated lymphocytes. IL-4 up-regulates the cytotoxic response but down-regulates the proliferation of CD3-AK cells. Generation of CD3-AK killer cells in resting lymphocytes is susceptible to the inhibition by PKC antagonist at non-toxic doses which does not significantly affect their proliferative response. The reverse is true for the preactivated CD3-AK cells.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0—864-939

Major Findings:

I. IL-4 regulation of a PKC-independent pathway for the generation of αCD3-induced activated killer cells. Depletion or inhibition of protein kinase C (PKC) by PKC depletor- or antagonist-induced unresponsiveness to the generation of αCD3-induced activated killer cells (CD3-AK) which were cultured in IL-2. The unresponsiveness was reversed by IL-4. These results were reproduced with purified T cells or purified $CD8^+$ cells. The phenotype of the killer cells were $Thy1^+$, $CD4^-$, and $CD8^+$. In the long term (2-week) PKC-depleted cultures, switching lymphokines reversed their cytolytic activity: switching from IL-2 to DL-4 restored cytolytic activity, and switching from IL-4 to IL-2 reduced cytolytic activity. The cytolytic activity of these CD3-AK cells correlated with their ability to produce BLT-esterase. These findings indicate that IL-4 may participate in the regulation of a PKC-independent pathway for the generation of CD3-AK cells by regulating the production of cytolytic granules.

II. Regulation by glutathione (GSH) of the activation of resting and preactivated lymphocytes. GSH added during first 24 hr decreased the generation of IL-2 dependent LAK and CD3-AK cells from resting lymphocytes, whereas after 48 hr of activation, the addition of GSH increased the killer cell activity. This dichotomous effect of GSH on the regulation of lymphocyte activation was due to the inhibition by GSH of signal transduction through IL-2 receptor or T cell receptor in primary response. However, once the lymphocytes were activated, intracellular GSH up-regulates the further differentiation of preactivated lymphocytes by up-regulating IL-2 utilization. Thus it appears that the balance between the activation signal and the immunoregulatory signal (induced by GSH) may determine the outcome of the immune response.

III. PKC and cytokine regulation of the function and production of cytolytic granules. In studying the role of PKC on the effector function of CD3-AK cells, it was found that PKC antagonists inhibited slow lysis but had no effect on fast lysis. These results indicate that fully activated $CD3\text{-}AK^+$ cells which can mediate fast lysis do not require PKC to mediate lytic reactions, whereas effectors on which mediated slow lysis ($CD3AK^-$ cells) required further activation and this process was PKC dependent. Furthermore, PKC antagonists inhibited the production of cytolytic granules, but did not directly affect the lytic activity of purified cytolytic granules. Il-4 reversed the inhibitory effect of PKC antagonists and restored the ability of CD3-AK cells to produce the cytolytic granules. These findings show that PKC and cytokines regulate the production but not the lytic activity of cytolytic granules.

IV. Differential regulation by cytokines and PKC of the proliferative and cytotoxic responses to αCD3 activation of resting lymphocytes and preactivated lymphocytes. IL-4 up-regulates the cytotoxic response but down-regulates the proliferative response to αCD3 activation of murine lymphocytes. In primary αCD3 sensitization, PKC antagonists used at non-toxic doses inhibited the cytotoxic response but did not significantly affect the proliferative response. In contrast, proliferation of preactivated CD3-AK cells was very susceptible to the

non-toxic doses, inhibited the cytotoxic response but did not significantly affect the proliferative response. In contrast, proliferation of preactivated CD3-AK cells was very susceptible to the inhibition by PKC antagonists whereas the cytolytic activity of preactivated CD3-AK cells was relatively resistant to the inhibition by PKC antagonists. The mechanism is being investigated.

Proposed Course of Study:

1. To study the regulation by PKC and cytokines of the production of cytolytic granules and expression of perforin gene in response to the activation of TCR-CD3.

2. To study the selective inhibition by various PKC antagonists of the expression of different PKC isozymes in lymphocytes.

3. To determine the role of different PKC isozymes in the regulation of various biological functions of lymphocytes.

4. To perform combined immunotherapy of murine tumor growth with CD3-AK, IL-4 and αCD3.

Publications:

Ting C-C, Hargrove ME. Anti-CD3 antibody-induced activated killer cells: Cytokines as the additional signals for activation of killer cells in effector phase to mediate slow lysis. Cellular Immunol 1991;135:273-84.

Ting C-C, Hargrove ME. IL-4 regulation of a protein kinase C independent pathway for the generation of αCD3-induced activated killer cells. Cellular Immunol 1992;140:130-44.

Ting C-C, Hargrove ME, Liang SM, Liang CM, Sharrow SO. Dichotomy of glutathione regulation of the activation of resting and preactivated lymphocytes. Cellular Immunol 1992; in press.

CONTRACT RESEARCH SUMMARY

Title: Provide Computer Programming Support Services for the Experimental Immunology Branch

Principal Investigator: Lorenzo F. Exposito
Performing Organization: SYSTEX, Inc.
City and State: Beltsville, MD

Contract Number: N01-CB-05689
Starting Date: 09/10/90 Expiration Date: 09/09/92

Goal: Perform computer programming support for the flow cytometry laboratory of the Experimental Immunology Branch. This support is required in order to make the transition from obsolete flow cytometric equipment and associated ADP hardware/software to state-of-the-art equipment supported by DEC/VAX/VMS ADP technology.

Approach: Work is performed in response to task orders for specific programming tasks which are determined by the Project Officer. Technical briefings are held to determine requirements and the contractor then designs, produces, installs, tests, and documents the required software.

Progress: Implementation under actual experimental conditions of software produced under this contract which interface with BDIS software for data acquisition, directory creation, network transfer and archiving of flow cytometry data. Further testing, modification and enhancement of multiple programs for data handling, hardcopy output, and system management including REPORT, UAF_MANGAGER, and HPGL plot software. 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 list mode data reduction capabilities for flow cytometry data. Continued testing of the tape carousel archiving system. Installed and configured uninterruptable power supplies for protection of VAX systems. Problem solving and interface with field engineers for multiple hardware breakdowns.

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 92 Funds: $73,419 D

CONTRACT RESEARCH SUMMARY

Title: Feral Mouse Breeding Colony

Principal Investigator: Ms. Evelyn Hogg
Performing Organization: Hazelton Laboratories America, Inc.
City and State: Rockville, MD

Contract Number: N01-CB-21055 (Successor to Contract CB-95261 which expired 11/31/91)
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.

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^+, and MS(M. spretus) mice. Three transgenic mouse lines containing the *Int-3*, *Wnt-1* and TGF alpha transgenes are also being maintained. These mouse strains are being used to determine the consequences of transgene expression on mammary gland development and mammary tumorigenesis. In addition, a limited breeding nucleus of the high-incidence C3H/OuJ and GR inbred mouse strains, the BALB/cfCzechII and BALB/cfMS mouse strains, and the low-incidence BALB/cP and FVB inbred mouse strains are maintained.

Progress: The contractor maintains the feral mouse colony in excellent condition, and carries out the breeding program, quality control, and maintenance of records in a highly satisfactory manner. More than thirty mammary tumors have been obtained from the high-incidence feral and inbred mouse strains. In addition, 10 CzechII mammary hyperplastic alveolar outgrowth lines have been developed and maintained to identify new cellular genes which are activated (inactivated) by MMTV insertional mutagenesis. One of these lines appears to have a viral insertion in the *Int-3* gene. The various transgenic mouse lines have been successfully bred and maintained. Analysis of female *Int-3* transgenic mice have provide several new insights into mammary gland development and the potential role of *Int-3* in mammary tumorigenesis. For instance the *Int-3* transgenic females are incapable of lactation due to arrested mammary gland development. In these mice there is limited ductal penetration of the female mammary fat pad and little or no development of alveolar structures. Moreover there is evidence of intraductal hyperplasia within which focal tumors arise. Currently bitransgenic mouse strains containing *Int-3* and TGF alpha; and *Int-3* and *Wnt-1* are being developed by genetic crosses.

Significance to Cancer Research: Provide essential support for the study of mammary tumorigenesis with the specific goal of identifying and characterizing the genes at risk to MMTV activation. Provides essential biological material for othe investigators studying the biology of the mouse mammary tumor virus as well as other classes of retroviral genomes.

Project Officer: Dr. Robert Callahan
Program: Immunology Resource
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group
FY 92 Funds: $112,991

CONTRACT RESEARCH SUMMARY

Title: Induction, Transplantation, and Preservation of Plasma Cell Tumors and Development of Special Mouse Strains

Principle Investigator: Judith Wax
Performing Organization: Hazleton Laboratories
City and State: Rockville, MD

Contract Number: NO1-CB-21075
Starting Date: 02-01-92 Expiration Date 01-31-97

Successor to Contract #NO1-CB-71085 which expired on 01/31/92

Goal: Induction, transplantation, preservation and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of (congenic) strains of mice to find genes controlling susceptibility and resistance to the induction of plasma cell tumors by pristane; maintence 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 ALB/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 plasmacytoma induction experiments using pristane alone in various BALB/c.DBA/2 congenic strains and hybrids of BALB/c and DBA/2. In addition, contractor has carried out plasmacytoma induction experiments in pristane conditioned mice with various retroviruses that carry oncogenes. Contractor transplants essential tumors and supplies tissues and/or DNA to investigators in the Laboratory of Genetics for molecular studies. Contractor ships mice, tumors, DNA, serum or ascites to other investigators. Contractor continues to develop essential congenic strains that are being used to identify new genes. Contractor prepares DNA and submits labelled samples to NCI for RFLP analyses. In addition to the above described work, the contractor functions as the major animal resource for the Laboratory of Genetics.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter, Dr. Beverly Mock
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 92 Funds: $903,322 Est. B

CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Norman Beaudry
Performing Organization: Hazleton Biotechnologies Corp.
Vienna, Virginia

Contract Number: NO1-CB-7-1010
Starting Date: 6/30/87 Expiration Date: 6/29/93

Goal: To perform radioimmunoassays of immunoglobulin molecules as well as ELISA assays of soluble interleukin-2 receptor molecules in lymphocyte culture supernatants or in biological fluids.

Approach: The contractor is to quantitate human immunoglobulins in various fluids using double antibody radioimmunoassay procedures and reagents defined and supplied by the project officer. In addition the contractor is to utilize an established ELISA assay for the soluble form of the IL-2 receptor to quantitate the level of this peptide in the serum of patients. Furthermore, the contractor is to measure antibodies to administered antigens and to murine and human monoclonal antibodies for the study of IL-2 receptor directed therapy of human neoplasia.

Progress: The contractor has established the required radioimmunoassays and the ELISA assays. Elevated IL-2 receptor levels have been demonstrated in the sera of patients with HTLV-I Adult T cell Leukemia, HIV Associated AIDS, Hairy Cell B Cell Leukemia, or Hodgkin's Disease. The assays for murine monoclonal antibodies as well as human anti-murine antibody responses has been developed and applied to the study of patients receiving IL-2 receptor directed therapy.

Significance to Cancer Research: These studies helped elucidate the abnormalities of the immune system associated with the development of cancer. They have assisted in the categorization of malignancies of the lymphoid system. They are required for therapeutic protocols involving the use of the anti-Tac monoclonal antibody. The studies of circulating IL-2R peptide levels are of importance in defining the biology of neoplasia, as an aid in diagnosis, assessment of prognosis, and in monitoring therapy of IL-2 receptor positive malignancies. The assays for antibodies to murine and human 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: 1992 Funds: $283,159, Est. B

CONTRACT RESEARCH SUMMARY

Title: Maintains an Animal Holding Facility and Provides Associated Research Services

Principal Investigator: Ms. Leanne DeNenno

Performing Orgainization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: N01-CB-85607
Starting Date: 11/01/87 Expiration Date: 10/31/93

Goal: Maintain colonies of up to about 10,000 inbred mice, 500 inbred rats, 50 hamsters, and 40 rabbits and carry out selected protocols with these animals as specified by the project officer. These animals are to be maintained in support of intramural research programs in the Experimental Immunology Branch, NCI.

Approach: Colonies of mice, rats, hamsters, and rabbits are to be housed and fed according to National Research Council standards. Technical manipulations and breeding are to be carried out as directed by the project officer.

Progress: This contract represents predominantly a facility for holding experimental animals in support of the research of the Experimental Immunology Branch. As such, the contractor has maintained colonies of up to about 10,000 mice, 500 rats, 50 hamsters, and 40 rabbits. Breedings of certain strains of mice for experimental needs have been performed when such animals have not been available commercially. Frozen samples of sera and cells, stored in freezers of appropriate temperatures, have been transferred to NIH as required.

Performance on this contract has been very satisfactory. The animal colonies are being maintained according to National Research Council standards. Animal health has, in general, been good. Protocols have been carried out in a satisfactory fashion. Record keeping and transferring of animals to and from the NIH Campus have all been satisfactory. Maintenance of frozen products in appropriate freezers has been satisfactory.

Significance to Cancer Research: This animal colony is necessary for support of intramural research programs in the Experimental Immunology Branch of NCI. Many of these programs are concerned with the immune response to cancer.

Project Officer: Dr. John R. Wunderlich
Program: Immunology Resource
Technical Review Group: Intramural Support Contract Subcommittee A
FY 92 Funds: $780,000 B

CONTRACT RESEARCH SUMMARY

Title: Facility for Preparing and Housing Virus Infected Mice, Genetically Manipulated Mice and Chimeric Mice

Principal Investigator:	Ms. Kinta Diven
Performing Organization:	Bioqual, Inc.
City and State:	Rockville, MD

Contract Number:	N01-CB-8-5608		
Starting Date:	09/30/88	Expiration Date:	09/30/93

Goal: Perform a variety of in vivo experiments in mice (up to a colony of 3600 animals) that cannot be performed on NIH campus as designated by the Project Officer. These experiments are to be performed in support of intramural research programs in the Experimental Immunology Branch, NCI.

Approach: Experiments are to be performed involving the transfer of normal and neoplastic cells, infection with virus, inoculations of combinations of cells and virus, irradiation with γ-rays, preparations of radiation chimeric mice, thymus transplants and the breeding, care, and manipulation of SCID mice. Protocols and details of experiments are to be carried as directed by the Project Officer.

Progress: Experiments have been performed that involve: irradiation and bone marrow transplantation, thymectomy, immunization, viral preparations bleeding, thymus and skin grafting, and breeding, care and manipulation of SCID mice. The following represent approximate numbers of tasks performed for FY'92: Mice received, 4400; mice born and weaned at contract site, 2300; radiation chimeras prepared, 880; bone marrow prepared from 720 mice, lymphoid cell preparations, 1260 preparations; i. p. inoculations, 5000; foot pad injections, 620; tail bleeds, 3500; palpations, 4100; ascities harvested, 470 ml; influenza A virus prepared, 800 ml.

Significance to Cancer Research: This experimental mouse facility is required to support the intramural research programs of the Experimental Immunology Branch of NCI in that it provides research that cannot be performed on the NIH campus due to animal restrictions and use of infectious agents in NIH animal colonies. All of the protocols used in the facility related to viral infection, genetic manipulation of hemotopoietic reconstitution of the immune system.

Project Officer: Dr. Gene Shearer
Program: Immunology Resource
Techinical Review Groups: Intramural Support Contract Subcommittee A
FY 92 Funds: $474,504 EST. B