

91 annual report

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Cancer Biology, Diagnosis, and Centers

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

ANNUAL REPORT

October 1, 1990 through September 30, 1991

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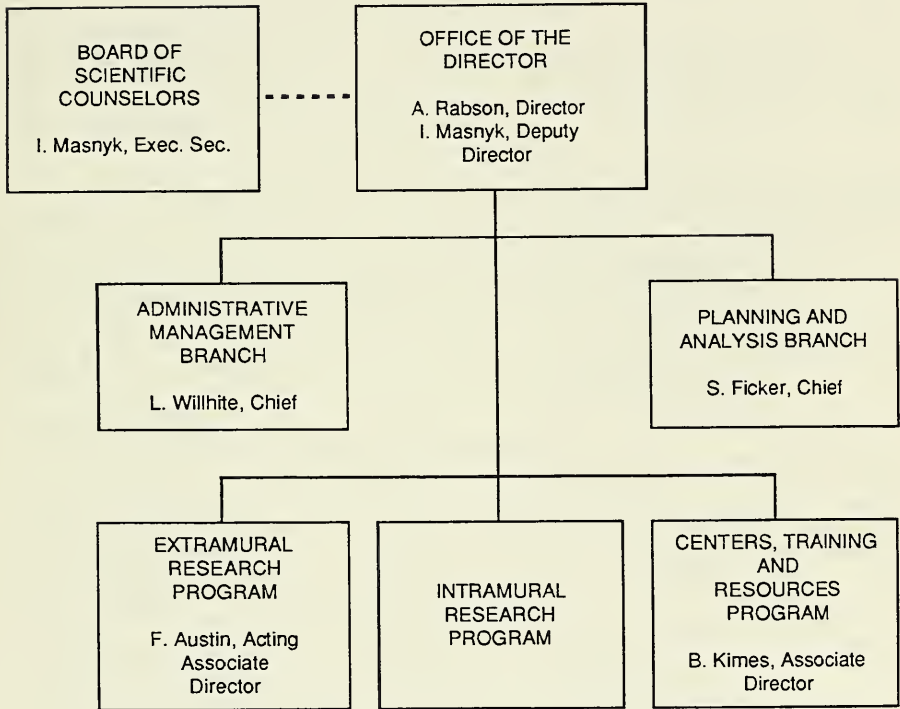
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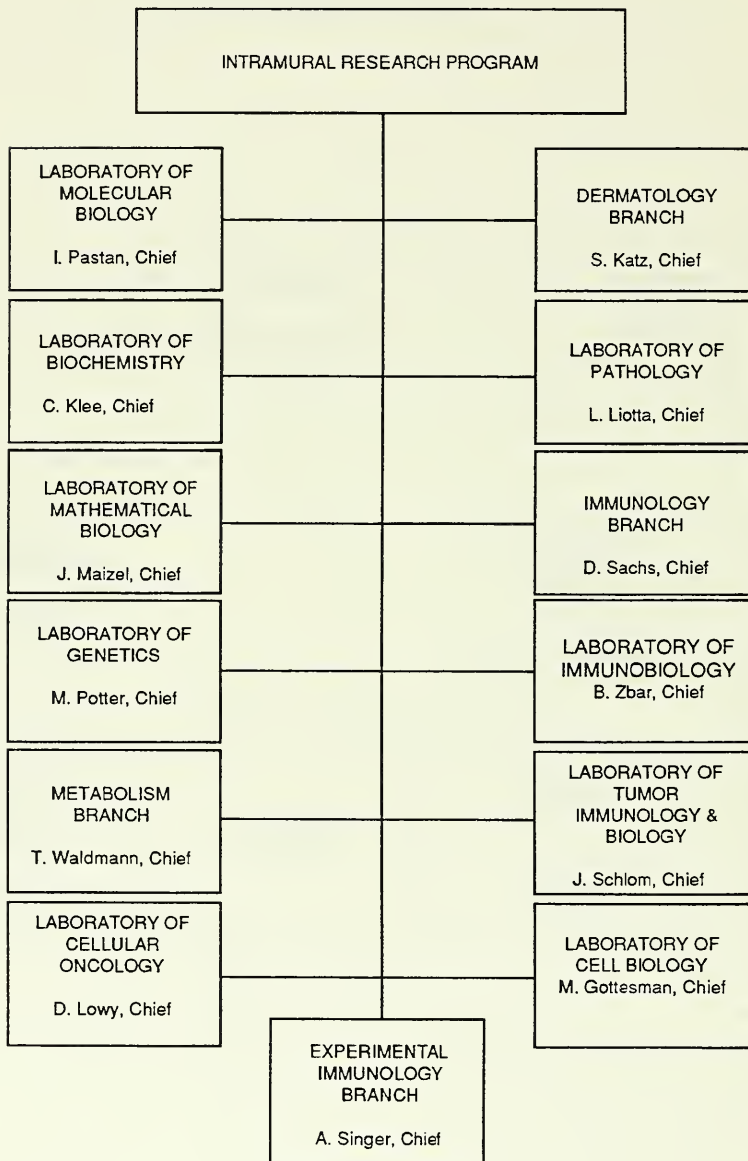
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**DIVISION OF CANCER BIOLOGY,
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NATIONAL CANCER INSTITUTE
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1990 through September 30, 1991

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 Resources Program, the Extramural Research Program, and the Intramural Research Program. 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 thirteen 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, the Immunology Branch, and the Laboratory of Tumor Immunology and Biology. Each of these laboratories is directed by a scientist of international stature, three of whom are members of the National Academy of Science.

Research conducted in the intramural laboratories covers a broad range of investigations from the regulation of gene expression in 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 elucidate the genetic alterations responsible for neoplastic transformation, with special emphasis on understanding the genetic alterations associated with breast cancer. 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 a tumor cell 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. In addition, basic studies of the normal immune response have led to important discoveries regarding the immune dysfunction associated with the progression from asymptomatic HIV infection to the development of AIDS.

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

LABORATORY OF GENETICS

A major focus of research activity in the Laboratory of Genetics, directed by Dr. Michael Potter, is on understanding the genetic changes that take place in the neoplastic transformation of hematopoietic cells. 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

The primary model system studied by this group is the paraffin-oil induced plasmacytoma in mice. The genetic constitution of these animals plays a role in the development of these tumors. BALB/Can mice are highly susceptible to developing plasmacytomas, while most other strains, e.g., DBA/2, are resistant. One of the major projects is to identify and characterize the several genes that determine susceptibility and resistance. Some of the genes of DBA/2 origin that determine partial resistance to plasmacytomagenesis are located on a 30 M segment on chromosome 4 between the IB-4 and Anf loci. BALB/c.DBA/2 congenic strains carrying portions of this segment of chromosome 4 have been developed. These congenic mice develop a much reduced incidence of plasmacytomas and far fewer plasma cell proliferative lesions (foci) during the latent period. The reduced formation of foci provides a phenotype for determining susceptibility and resistance more rapidly than by tumor induction. Dr. Potter and his colleagues are searching for correlative phenotypes and have evidence that both DBA/2 and the two chromosome 4 congenics, C.D2-Fv-1^{n/n} and C.D2-Tol fam-3, repair UV induced DNA damage to the 5' end of the c-myc oncogene more efficiently than BALB/Can mice. They are attempting to localize Pctr (resistance) genes on chromosome 4.

Dr. Beverly Mock has injected BALB/c x (BALB/c x DBA/2)F1 1st generation backcross mice with pristane and induced the expected 11-13% plasmacytomas. She has genotyped these tumors for a variety of polymorphic genes and found linkage of susceptibility to the chromosome 4 markers Lsl, Ifa, D4Lgm3, Scl, D4Rpl and D4Lgml.

Organization and Control of Genetic Material in Plasmacytomas

The deregulation of the c-myc oncogene is a critical event in plasmacytomagenesis that is caused by chromosomal translocation. The molecular mechanisms of this deregulation are not yet understood. Some of these myc-activating translocations occur 200-300 kb 3' of c-myc in a region called Pvt-1. Drs. Fred Mushinski and Konrad Huppi have shown that this locus is transcribed at very low levels in normal cells, but in much higher amounts in some plasmacytomas. Normally Pvt-1 transcripts are ca. 14 kb in length, suggesting that the Pvt-1 locus is very large. In some plasmacytomas with chromosomal translocations in the Pvt-1 locus, the Pvt-1 transcripts are truncated and form chimeras with Ig kappa chain transcripts. Drs. Huppi and Mushinski are attempting to define the Pvt-1 locus and have identified new, distant exons. The function of Pvt-1 remains obscure.

Dr. Mushinski has isolated a cDNA for mouse bcl-3 (on human chromosome 19) and has mapped it to mouse chromosome 7. During B-cell differentiation its expression is maximal in cells that have not undergone Ig isotype switch. This suggests that the chromosome translocations involving this locus that characterize some chronic lymphocytic leukemias (CLL) occur at this stage.

Normal and Neoplastic Lymphocyte Development

Dr. Stuart Rudikoff has been studying the biological basis for DBA/2 resistance to pristane-induced plasmacytomagenesis by utilizing a series of transfer experiments introducing either BALB/c or DBA/2 bone marrow into SCID mice followed by tumor induction protocols. Tumor induction has employed either classic pristane treatment, or the use of a retroviral construct, J3V1, supplying deregulated myc and raf oncogenes. These studies indicate that SCID mice reconstituted with BALB/c bone marrow and injected with J3V1 develop plasmacytomas and myeloid tumors. In contrast, DBA/2 reconstituted SCIDs develop only myeloid tumors indicating that the genetic difference between the two strains, in terms of plasmacytoma induction, resides in the DBA/2 B-cell and that resistance cannot be overcome by the introduction of a deregulated myc oncogene which seems to be critical for induction in BALB/c.

Molecular and Biological Basis of Immune Response

Protein-protein interactions underlying molecular recognition are being studied using antibodies specific for the protein hen eggwhite lysozyme (HEL). During the past year, the X-ray structure of a third HyHEL-26 Fab-HEL complex was solved and is now being refined. The HyHEL-26 epitope is nearly identical to that recognized by HyHEL-10, and the primary and genetic structures of HyHEL-26 are closely related to those of HyHEL-8 and -10. These results represent the first time structures have been obtained for two structurally related antibodies complexed to the same protein epitope, thus providing an unprecedented system for studying structure-function relationships. Dr. Sandra Smith-Gill has begun to experimentally investigate structure-function relationships in the interface by site-specific mutagenesis of both antigen and antibodies. These studies are beginning to define fundamental principles that will allow prediction of function from structure, principles that are critical to such applications as anti-protein antibody design and vaccine development. Dr. Smith-Gill is also approaching the problem of vaccine development by investigating immunogenicity and protective epitopes in *Shigella flexneri*.

LABORATORY OF BIOCHEMISTRY

Research in the Laboratory of Biochemistry, directed by Dr. Claude Klee, continues to be diverse, reflecting the interests of several independent groups, but during the past year the overlap in approaches and techniques has become more evident. Successful expression of the transcription factors involved in the regulation of gene expression has facilitated the study of these factors as proteins. Collaborative studies have been initiated to determine the structures of these factors or of their active fragments by multidimensional nuclear magnetic resonance (NMR) spectroscopy. There is also a growing interest in the potential regulation of these factors by protein phosphorylation.

Regulation of Gene Expression

Dr. Carl Wu and his colleagues have continued to study transcription factors regulating heat shock genes and the segmentation gene *fushi tarazu*, ftz, in *Drosophila*. Three transcription factors: Heat Shock Factor (HSF), FTZ-F1, and FTZ-F2 (now referred to as tramtrack) have been expressed in *E. coli*, and the recombinant proteins have been characterized biochemically and used for the preparation of antibodies. Immunostaining has revealed the subcellular

distribution of the transcription factors and their pattern of expression in the developing embryo. A major accomplishment this year was the successful cloning of a human heat shock transcription factor. Analysis of the cloned human HSF gene revealed the existence of a fourth, C-terminal leucine zipper motif in the Drosophila and human proteins which is absent in the yeast protein. This fourth zipper motif may be involved in the regulation of HSF DNA-binding activity, which is heat shock inducible in Drosophila and human, but not in yeast. The cloning of Drosophila and human HSFs opens the way for a comparative molecular dissection of the heat shock response. A second highlight is the immunolocalization of the HSF protein to chromosomal puff sites of the Drosophila polytene chromosomes. In addition to the known heat shock loci, HSF is found at another 100 loci or so the Drosophila genome. This finding may define new genes that are targeted for HSF control. Dr. Wu's group has also developed a highly efficient, in vitro, chromatin reconstitution system from early Drosophila embryos which will be very useful for future studies of the interaction of transcription factors with the chromatin template.

Dr. Dean Hamer's group, in their continuing studies of gene regulation by metals, made the surprising discovery of an enzymatic reduction of copper. This process is highly regulated and appears to play a central role in the growth-promoting and repressing activities of this metal. They have also obtained detailed structural information on a yeast metalloregulatory protein and identified new human and mouse metallothionein gene transcription factors.

While continuing to study the structure, regulation and mode of action of a group of genes involved in determining the muscle phenotype in vertebrates, Dr. Bruce Paterson and his colleagues have initiated similar studies in Drosophila to take advantage of Drosophila genetics to facilitate identification of the physiological role of the factors encoded by these genes. They have isolated two chicken myogenic factor genes, CMD1 and Cmg1, which are homologues of mouse MyoD and myogenin, and a Drosophila homologue Dmyd. Each of the factors encoded by the members of this gene family shares a structural motif that is essential for function. Chimeric proteins that contain specific regions from the avian and Drosophila proteins are being used to identify regions responsible for the different activities. The regulation of these factors by protein phosphorylation is also being investigated.

Dr. Charles Vinson, who recently joined the laboratory, is studying the class of DNA-binding proteins involved in muscle cell determination. He has demonstrated that one protein of this class binds to two cis elements in a cooperative fashion, and he proposes that the interacting domain map to Helix II of the consensus sequence.

The role of DNA methylation in the modulation of gene expression continues to be the major focus of interest of Dr. Edward Kuff's laboratory. His group has been studying the regulation of an envelope-deficient mouse retrovirus, the intracisternal A-particle (IAP). Expression requires demethylation of sequences within the IAP 5' LTR and a constellation of trans-acting factors.

Organization of the Human Genome

Dr. Maxine Singer and her colleagues continue to study the mechanism of LINE-1 transcription in the human genome. They have shown that both transcription and translation of the human transposable element LINE-1 (L1s) have unusual

features. Cell-type-specific transcription of LHs mRNA is regulated by internal cis-acting motifs including an orientation-independent enhancer. Evidence is also accumulating for internal initiation of translation of both open reading frames.

Dr. Wesley McBride has developed several highly successful collaborative projects designed to determine the chromosomal localization of a large number of cloned genes. During the past year, 15 genes have been mapped. Progress is under way to isolate a series of highly polymorphic probes on chromosome 22 to be used to construct a high resolution genetic linkage map and physical map of human chromosome 22.

Proteins and the Control of Cellular Processes

Several projects in the laboratory deal with the role of enzymes and other proteins in the regulation of cell function. The Ca^{2+} -binding protein, calmodulin, plays a pivotal role in the Ca^{2+} regulation of several critical cellular events. During the past year, Dr. Claude Klee and her colleagues have concentrated their efforts on the Ca^{2+} and calmodulin stimulation of the protein phosphatase, calcineurin. Calcineurin is under a dual Ca^{2+} control mediated by an integral subunit of the enzyme, calcineurin B, and by calmodulin. The high affinity Ca^{2+} sites of calcineurin B, fully occupied at resting levels of Ca^{2+} , are postulated to play a structural role. The Ca^{2+} dependence of calmodulin stimulation is highly cooperative and dependent on the concentration of free calmodulin. In related work, Dr. Frank Supryniewicz, with continuing support from Dr. Klee, has demonstrated that the concerted action of a protein kinase(s) and a phosphatase(s) is needed to inactivate the mitosis-specific, cdc2 kinase and thereby to shut off mitosis.

Dr. Shelby Berger and her colleagues are studying the role of prothymosin α in cell division using antisense oligomers directed against several regions of prothymosin α mRNA. They are also studying the mechanism of action of the human immunodeficiency virus (HIV) reverse transcriptases. A complex composed of two ribonucleosides and one oxovanadium ion has been characterized as a potent inhibitor of the enzymes. With this inhibitor Dr. Berger's group provided strong evidence that a single DNA:RNA substrate binding site serves both the polymerase and the ribonuclease H functions.

DNA Replication

Studies of P1 plasmid maintenance by Drs. Michael Yarmolinsky and Dhruba Chattoraj focus on replication control, active partition and the lethal addition of bacteria to a resident P1 plasmid. These studies have established the DNA sequence features essential for RepA binding to its targets, provided evidence that RepA binding to the origin region is facilitated by heat shock proteins DnaJ, DnaK and GrpE and shown that this binding absorbs a positive superhelical turn of origin DNA.

Dr. Samuel Wilson and his colleagues have continued to take advantage of the bacterial overexpression of mammalian DNA polymerase β , A1 hn RNP single strand-binding protein and HIV-1 reverse transcriptase to pursue their studies of DNA replication. They have also made significant progress in the study of the structure-function relationship of HIV reverse transcriptase. The domain

responsible for the interaction between the primer substrate and the enzyme has been identified by UV-crosslinking of the oligo (dT)-enzyme and complex and subsequent sequencing of V8 protease peptides. Information on the properties of this substrate binding domain may make it possible to design more specific inhibitors of HIV-1 reverse transcriptase.

LABORATORY OF MOLECULAR BIOLOGY

The Laboratory of Molecular Biology, directed by Dr. Ira Pastan, 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 Oncotoxin Therapy of Cancer

Drs. Pastan, David FitzGerald, and Mark Willingham have been engaged in a major effort to construct new cytotoxic agents for the treatment of cancer and other diseases by fusing cell targeting genes to genetically modified forms of *Pseudomonas* exotoxin gene. This is done by fusing DNAs encoding growth factors, single chain antibodies, or other cell recognition molecules to DNAs encoding modified forms of *Pseudomonas* exotoxin. TGF α -PE40, which kills cells with EGF receptors, is being developed by Merck, Inc., for the therapy of bladder cancer; clinical trials are planned for late this summer. IL2-PE40 is very effective in killing rodent cells with IL2 receptors, but is much less active against primate and human cells. Several modifications have been made to produce a more active reagent. The most active of these is a single chain immunotoxin anti-Tac(Fv)-PE40 which is extremely cytotoxic to human and primate cells expressing IL2 receptors, including leukemic cells from patients with adult T-cell leukemia. A clinical trial is being planned. IL6-PE40 and a variant IL6-PE66^{401u} are cytotoxic to several myeloma cell lines and to hepatoma cell lines. Because tumors are dependent on new blood supply, a new construct has been developed employing acidic fibroblast growth factor (FGF) to target either PE40 or PE66^{401u}. These agents are cytotoxic to FGF receptor-bearing cells and are now undergoing preclinical evaluation. Last year it was reported that CD4-PE40 kills HIV infected cells by binding to the gp120 present on the surface of these cells. It has been shown that the combination of AZT and CD4-PE40 is synergistic and will arrest the spread of HIV infection in cultured cells and sterilize infected cell cultures. CD4-PE40 is being prepared by Upjohn and will enter clinical trials in the summer of 1991. A novel monoclonal antibody (B3) that is reactive with many colon, breast and ovarian tumors has been isolated. In addition, the genes encoding the variable regions of this monoclonal antibody have been cloned and fused to PE40 to create B3(Fv)-PE40. This single chain immunotoxin has been shown to have a striking antitumor effect against human tumors growing in nude mice. Several mutant forms of PE have been synthesized which have increased cytotoxic activity. PE is a very immunogenic molecule. Recent studies have shown that an immunosuppressive agent, 15-deoxyspergualin, can completely suppress the primary antibody response to PE in mice. It was previously shown that a 37 Kda fragment of PE containing the ADP ribosylating domain is translocated into the cytosol. Further studies have shown that PE can be used to introduce foreign peptides into the cytosol of cells for various purposes including for presentation to the MHC class I system.

Multidrug Resistance

In a collaborative project with Dr. Michael Gottesman, chief of the Laboratory of Cell Biology, Dr. Pastan has been investigating the molecular genetic mechanisms responsible for the multidrug resistance phenotype in tumor cells. The simultaneous resistance of cancer cells to many different anticancer drugs is the major impediment to successful chemotherapy of metastatic disease. An important mechanism of multidrug resistance is expression of P-glycoprotein, a 170,000 dalton energy-dependent drug efflux pump which removes natural product drugs from the cell. Their studies have shown that ATP is the preferred energy source. Molecular manipulations have identified the first intracytoplasmic loop as a domain involved in drug recognition, which is distinct from the drug-labeling sites. In collaboration with Dr. Glenn Merlino, a *MDR1* transgenic mouse has been developed whose bone marrow is protected from the cytotoxic effects of anticancer drugs by expression of P-glycoprotein. New in vitro models of resistance to chemotherapeutic agents such as VP-16 and cis-platinum, not involving the multidrug transporter, are under development.

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

In addition to the *MDR1* transgenic mouse, Dr. Merlino has also developed transgenic mice bearing either the human TGF α or EGF receptor gene. TGF α overexpression induces hepatocellular carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and gastric cystic hyperplasia. Transgenic mice made using an activated form of a related gene, int-3, which contains EGF repeats and is a member of the Notch gene family, developed hyperplasia of secretory epithelia and neoplasia of the salivary and mammary glands. In addition, male mice were sterile and female mice could not lactate.

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

Dr. Sankar Adhya has shown that each of the two promoters of the *gal* operon is negatively regulated by two repressors, GalR and newly discovered GalS. The repression by GalR is strong, whereas that of GalS is moderate, although both act by binding to the same two spatially separated operators, O_2 and O_1 .

The biochemical mechanism of negative control of the *gal* operon by GalR repressor has been studied in detail. Dr. Adhya has proposed that repression involves "caging" of RNA polymerase by a DNA loop formed by interaction of O_2 and O_1 bound repressors facilitating a transcription-inhibitory physical contact(s) between repressor and RNA polymerase. Consistent with the model, Dr. Adhya has shown that 1) the specificity of the contact between operator-bound repressors is independent of the nature of DNA-protein interactions and resides in the carboxy domain of the repressor protein, 2) the proposed repressor-RNA polymerase contact may be through the α -subunit of the holoenzyme, and 3) transcription from both *gal* promoters in a unitary promoter plasmid minicircle is repressed in a purified system with wild type repressor. Repression was not observed with a mutant repressor that binds to the operators, but does not loop the DNA.

Bacterial Functions Involved in Cell Growth Control

Dr. Susan Gottesman has continued to study the role that protein degradation plays in regulating gene expression and has initiated new studies on the linkages

between chromosome synthesis and partition of chromosomes during cell division. The mechanism of regulation of capsule synthesis serves as a model for understanding the role of unstable proteins as regulators. RcsA, a target for the Lon ATP-dependent protease, was shown to interact with another regulator, RcsB, in a temperature sensitive manner. Studies on *rcsA* expression have demonstrated a trans-acting role for a site downstream of the structural gene in regulating *rcsA*. Studies are in progress on a new proteolytic activity capable of degrading the Lon substrates, RcsA and SulA, which appears when a multicopy plasmid carrying a gene (*alp*) is introduced into cells. The new activity is dependent on excision of a cryptic prophage and consequent inactivation of a chromosomal gene encoding a stable RNA. A third energy-dependent protease under study is the two component Clp protease. Mutagenesis studies of the ClpP subunit are ongoing to determine the specificity and the role of ClpP-dependent ClpP processing for assembly and proteolytic activity.

LABORATORY OF CELL BIOLOGY

The primary research focus in the Laboratory of Cell Biology, directed by Dr. Michael Gottesman, is on the molecular genetic analysis of the multidrug resistance phenotype of certain cancer cells. Other projects include studies of the effects of cAMP and p53 on regulation of cancer cell growth, the role of surface antigens and proteases in metastasis, the mechanism of energy-dependent proteolysis, and the mechanism of antigen processing.

Resistance of Cancer Cells to Anti-Cancer Drugs

The major mechanism of intrinsic and acquired resistance to multiple natural product chemotherapeutic drugs is the expression of the *MDR1* gene, which encodes a 170,000 dalton membrane glycoprotein which acts as an energy-dependent drug efflux pump (P170, P-glycoprotein, or multidrug transporter). Collaborative studies by Dr. Gottesman and Dr. Pastan have revealed that there are two labeling sites on the transporter, believed to represent the two halves of the transporter which come together to form a "channel" through which drugs move, and that the first intracytoplasmic loop of the transporter contains information involved in drug recognition. Basic recognition of multiple, chemically unrelated substrates appears to occur within the plasma membrane.

An in vivo model system, in which the human *MDR1* cDNA is expressed in the bone marrow of transgenic mice, has shown that levels of expression of *MDR1* RNA comparable to those found in many human cancers are sufficient to confer drug resistance on bone marrow. This model has allowed the testing of many potential inhibitors of the multidrug transporter. Functional drug resistant bone marrow may be transferred from resistant to sensitive animals, demonstrating the potential utility of the *MDR1* cDNA as a dominant selectable marker in human gene therapy experiments. Retroviral vectors which allow expression of the human multidrug transporter in transformed bone marrow and muscle provide models for protecting normal human tissues against the toxic effects of chemotherapy, and for the use of the *MDR1* cDNA in co-transformation of non-selectable genes.

Synthesis and Function of a Transformation-Dependent Secreted Lysosomal Protease

Cathepsin L is a major cysteine acid protease which is secreted by many rodent and human tumor cells. Dr. Gottesman's group has shown that human renal cancers,

testicular cancers, and non-small cell lung cancers have especially high levels of cathepsin L mRNA, indicating that it may be a useful biological marker for the presence of these cancers, and a potential site for anti-cancer therapy. Regulation of the mouse cathepsin L gene is complex, with regulatory elements present both "upstream" and "downstream" from the start site of transcription.

The Mechanism of Energy-Dependent Proteolysis

ATP-dependent proteases are responsible for a major portion of the degradation of intracellular proteins in eukaryotic and prokaryotic cells, and play vital roles in cell division, adaptation, stress responses, and normal cell maintenance. The *E. coli* Clp protease is representative of a family of ATP-dependent proteases found in all organisms. Dr. Michael Maurizi and his colleagues have focused their efforts on the biochemistry of proteolysis by Clp protease and on the identification of the physiological targets of the enzyme.

The Mechanism of Antigen Processing

Research in Dr. Ettore Appella's laboratory has emphasized the use of techniques in peptide and nucleic acid chemistry. Of particular interest is his work on the synthesis and expression of peptides derived from the HIV virus. He has synthesized peptides which mimic the binding of a cellular transcription factor to regulatory sequences in HIV long terminal repeats (LTRs). These studies represent an initial effort to understand the structural and mechanistic features of regulation of HIV transcription and to design strategies to interfere with HIV expression.

LABORATORY OF CELLULAR ONCOLOGY

The Laboratory of Cellular Oncology, directed by Dr. Douglas Lowy, plans and conducts fundamental research on the cellular and molecular basis of neoplasia.

Oncogene Expression In Vitro and In Vivo

Dr. Lowy and his colleagues have continued their study of ras-encoded proteins. They analyzed chimeras between ras and rap-1A, which encode a ras-like protein that can suppress ras-transformed cells. The results indicated that the respective effector regions of ras and rap-1A determined whether the protein induced cellular transformation or suppressed transformation, which suggest that rap-1A may suppress ras-induced transformation by interfering with the interaction between ras protein and its effector. Further analysis revealed that distinct amino acids of p21ras and p21rap-1A mediate sensitivity to each of the proteins with GAP activity, and that ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap-1A, respectively, in NIH 3T3 cells.

Analysis of Papillomaviruses

Certain types of human papillomaviruses (PHVs) are frequently detected in human genital cancers, and therefore are designated high-risk types, while other low-risk types are frequently detected in benign (but not malignant) genital lesions. Dr. John Schiller and Dr. Lowy have undertaken a comparative analysis of the gene products of high risk and low risk viruses in an attempt to gain insight into the important determinants of pathogenicity for the genital HPVs. They have

determined that the E6s and E7s of the two viral classes differ in their ability to induce cellular transformation and immortalization. E6 and E7 also activate transcription of test promoters, but there does not appear to be a correlation between trans-activation and oncogenic potential. They have also determined that the E7s of high- and low-risk types differ in their ability to be expressed from polycistronic mRNAs.

The mechanisms of E6 and E7 induced transformation and immortalization are under investigation. For E6 they have determined that the full-length protein product, but not the truncated E6' proteins unique to the high risk types, is required for these activities. Additional studies indicated that mutant p53 can functionally substitute for E6 (but not E7) in the immortalization of normal human keratinocytes, but not in the transformation assay, suggesting that an interaction with wild type p53 may be important for immortalization, but that a separable E6 function may be required for transformation. For E7 they are currently identifying the cellular proteins with which E7 interacts and evaluating the biological significance of these interactions.

LABORATORY OF IMMUNOBIOLOGY

The Laboratory of Immunobiology, directed by Dr. Berton Zbar, conducts research in two major areas: genetic studies of human renal cell carcinoma, and basic studies of the inflammatory response.

Genetic Basis of Human Renal Cell Carcinoma

Von Hippel-Lindau disease (VHL) is an autosomal dominant, multisystem neoplastic disorder. Last year it was shown that the VHL gene was isolated on the short arm of chromosome 3 in a 6-8 Cm interval between RAF1 and D3S18. During the past year efforts have focused on the isolation and characterization of new probes from human chromosome 3. As part of this effort, 41 families with VHL have been evaluated. One large family was identified whose disease phenotype was distinct from typical VHL. The manifestations in this atypical family were linked to RAF1 and to D3S18. These results suggest that there are mutant alleles or several contiguous genes (at least three) at the VHL locus associated with distinct tissue specificities. A marker, D3S601, located between RAF1 and D3S18 has been identified. It is the closest marker to VHL that has been identified and is located between proximal and distal breakpoints in the region immediately surrounding the VHL gene. In addition, Dr. Zbar has shown that DNA-polymorphism analysis can identify individuals likely to carry the VHL disease gene among asymptomatic members of disease families.

In a collaborative effort, 43 probes have been identified that are homozygously deleted in a small cell lung carcinoma line. This is of interest because previous studies showed that regions of the genome that are homozygously deleted in this cell line usually contain tumor suppressor genes. Studies have also shown the human squamous cell carcinoma of the head and neck is characterized by a loss of alleles at loci on chromosome 3p. Work supported by a grant from the National Center for Human Genome Research has enabled progress in the preparation of a high resolution genetic map of human chromosome 3p.

The Role of Chemotactic Factors in Inflammatory Response

Dr. Edward Leonard's studies of the inflammatory response continue to focus on three human host defense proteins--neutrophil attractant protein-1 (NAP-1), monocyte chemoattractant protein-1 (MCP-1), and macrophage stimulating protein (MSP). Now that NAP-1 and MCP-1 have been cloned and sequenced, the major objective is to determine biological significance. Reagents and new assay systems have been developed which should aid in these studies.

LABORATORY OF MATHEMATICAL BIOLOGY

Research in the Laboratory of Mathematical Biology, directed by Dr. Jacob Maizel, covers a broad range of theoretical and experimental studies of biological systems. Application of the basic understanding of these biological systems, serving as models for aspects of the cancer and other process, is accomplished through the use of advanced computing. The Laboratory often develops computational and experimental methodology that is utilized by researchers in the biomedical community at large. Many of the theoretical studies have only been possible through the use of supercomputing facilities at the Advanced Scientific Computing Laboratory, Frederick Cancer Research and Development Center (FCRDC).

Sequence Analyses in Virology, Cell and Molecular 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. RNA structures up to 2000 bases in size have been predicted. Methods to assess the significance of predictions have used Monte Carlo simulations, evolutionary comparisons and biochemical data. New sequences were compared with computerized databases to detect relationships with known proteins.

RNA secondary structure methods have been refined by Dr. Maizel and his colleagues 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 mRNAs. The power of the supercomputer has allowed development of a lookup procedure for predicting stability of random sequences, which accelerates surveys nearly 100-fold. Monte Carlo techniques are being developed that yield greater than 80% correct prediction of t-RNA structures for more than 100 examined sequences.

Reasonable three-dimensional models for perforin, apo-lipoprotein and parts of HIV reverse transcriptase were built by computer methods. Biochemical data is testing these predicted models.

Information Theory in Molecular Biology

Dr. Thomas Schneider's group is using information theory 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. 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.

Molecular Structure

Another research project is directed towards studying the properties of biological macromolecules, including peptides, proteins, DNA and RNA, as a model for studying even larger molecular assemblies. One principal difficulty in achieving the correct folded conformation of a protein is the overwhelmingly large number of possible conformations. Dr. Robert Jernigan's group has developed a strategy to overcome this difficulty that involves restricting the space to the overall size and shape, for conformation generation, to afford 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. 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. In a similar approach, studies have begun on tertiary folding of RNA and on investigating the binding of small peptides to larger proteins.

Molecular Modeling

Molecular modeling has been proceeding in four areas: membrane proteins, small peptides, DNA helices, and DNA-protein interactions. For the membrane proteins, conformational models have been developed for the antibiotic magainin, delta lysin, and cecropins. These models have improved our understanding of how they lyse cells and form channels. Models have also been developed for three groups of channel proteins, voltage-gated potassium channels, annexins, and paradaxin. Small peptide models have been built on the basis of 2D NMR data indicating close atoms and molecular calculations. 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. Other DNA forms, such as three-stranded helices and alternative base pairs, are also being modelled.

Theoretical Immunology

Studies in Dr. John Weinstein's group are aimed at understanding the physiology and pharmacology of biological ligands to aid in the rational design of next-generation molecules for treatment of cancer and AIDS. Recent work has centered on quantitative modeling of the pharmacology of monoclonal antibodies.

Dr. Weinstein and his colleagues have continued to develop a new approach for combination chemotherapy of HIV infection. Previous studies demonstrated that that Dipyridamole (Persantin), widely used for cardiovascular indications, potentiates the activity of AZT and other dideoxynucleosides against HIV in monocytoid and stimulated T-lymphocytes. In fact, 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. 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) are in progress.

Membrane Structure and Function

Dr. Robert Blumenthal's research program is directed towards elucidating the mechanisms of membrane fusion mediated by viral glycoproteins. Of particular interest has been studies of the mode of action of the envelope protein of HIV. The initial steps of HIV envelope protein-mediated membrane fusion have been studied by continuous monitoring of fluorescent dyes during fusion using fluorescence spectroscopy and low light, image enhanced videomicroscopy. HIV envelope protein expressed in cells by means of recombinant vaccinia virus and target membranes of defined composition with and without CD4 receptors are studied to 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 is 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.

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 and cytopathology 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.

Pulmonary and Postmortem Pathology

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.

A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome 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

The Cytopathology Section provides diagnostic service on exfoliative cytology, fine needle aspiration cytology, and immunocytochemistry. Dr. Diane Solomon's research program has 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. Currently they are investigating the use of *in situ* hybridization as an ancillary diagnostic technique.

Ultrastructural Pathology

The Laboratory of Pathology provides diagnostic electron microscopy services for the NIH Clinical Center. Dr. Maria Tsokos conducts an active research program in the characterization of small round cell tumors of childhood. These tumors comprise one of the most difficult groups of tumors in the differential diagnosis of cancer in children and young adults. Dr. Tsokos has focused on 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 the definition of histologic, biologic and other factors to predict biologic aggressiveness.

Tumor Invasion and Metastases Section

A major focus of experimental studies, directed by Dr. Liotta, is on elucidating the molecular genetic changes that occur when a tumor cell acquires the ability to invade and metastasize. Expression of the metastatic phenotype depends on a balance between positive and negative regulatory gene products. Understanding the action of these gene products has led to new strategies for prognosis and therapy.

Dr. William Stetler-Stevenson is studying type IV collagenase, a metalloproteinase first identified in this laboratory, 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 may function as a tumor suppressor protein by inhibiting metalloproteinase activity required for invasion. *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 has obtained the full-length cDNA for NM23, a putative tumor suppressor gene that is associated with invasiveness. She has identified the 17 Kda protein product of this gene and found that it is conserved in evolution. 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 in rodent melanoma models. Recent studies indicate that the NM23 protein is an NDP kinase. 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. The gene for a potent motility stimulating cytokine, AMF, and the gene for a new transmembrane

protein which regulates tumor cell locomotion have been cloned. A signal transduction inhibitor which blocks tumor cell cytokine stimulated growth and motility has been identified. The inhibitor, termed CAI, is a substituted imidazole which constitutes a new approach to cancer therapy. CAI has produced primary tumor and metastasis regression following oral administration in animal models. CAI has low toxicity and is being considered as a potential chemopreventive agent. Clinical phase I trials are scheduled to begin by the end of 1991.

Hematopathology

Dr. Elaine Jaffe directs a major program in diagnostic and experimental hematopathology. Her research focuses on the immunological characterization of malignant lymphomas. She has 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, and none of the patients developed disseminated large cell lymphoma. Dr. Jaffe's group 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 (EBV). 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.

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 the sensitive PCR technique, she can detect lymphoma cells which were otherwise undetectable in clinical samples.

Dr. Mark Raffeld has completed a molecular analysis of small non-cleaved 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 non-cleaved cell lymphoma. He has recently completed a collaborative study of the molecular genetics of gastrointestinal non-Hodgkin's lymphomas. This study found a low incidence of bcl-1 and bcl-2 translocations, suggesting a different pathogenesis for gastrointestinal non-Hodgkin's lymphoma. In a similar study, the patterns of c-myc rearrangements in gastric large cell lymphoma and ileocecal Burkitt's lymphoma suggest a different and distinct pathogenesis for these two aggressive lymphomas.

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: 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.

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 on five clones have been completed and have revealed two functional classes of proteins encoded by these genes: lymphokines and DNA binding proteins/transcription factors. Potential functional activities of the three putative lymphokines currently are being tested with recombinant proteins.

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 Branch also serves as Dermatology Consultant to all other services of the Clinical Center.

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases

Dr. Stephen 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). The LC express greatly increased amounts of class II MHC on their surfaces and become potent antigen-presenting cells. In addition, Dr. Katz is using a sensitive reverse transcriptase PCR technique to assess changes in epidermis-derived cytokine mRNA levels early in the afferent phase of contact sensitivity to identify potentially important LC and keratinocyte alternatives. The studies to date demonstrate that LC-derived and certain keratinocyte-derived cytokine mRNAs are selectively upregulated by allergens in the very early afferent phase of contact sensitivity. They are now assessing the role of physical agents (i.e., UV), as well as other chemical agents and even infectious agents (i.e., H. Simplex and HIV) to affect epidermis-derived cytokines and thereby affect inflammatory and immune responses in the skin.

Molecular Basis of Autoimmune Skin Diseases

Dr. John Stanley's group is studying autoantibody-mediated skin diseases in order to better understand not only the pathophysiology of these diseases, but also the structure and function of normal epidermis and epidermal basement membrane zone. Bullous pemphigoid (BP) is known to be a component of the hemidesmosome, a basal cell-substrate adhesion junction. They have determined that the BP antigen is a 230 Kd protein with a Pi of 8, and have begun using the PCR for the rapid amplification of cDNA ends (RACE) to finish cloning the 5' end of the BP cDNA. In addition, they have begun characterization of the BP antigen gene in normal humans, animals, and patients with junctional epidermolysis bullosa, a disease with abnormal hemidesmosomes. Using immunochemical methods they have defined a new form of pemphigus, paraneoplastic pemphigus, that is clinically, histologically, and molecularly unique and is associated with lymphoma. Finally they have started cDNA cloning of the pemphigus vulgaris (another antibody-mediated disease) antigen.

Therapy of Skin Cancer and Disorders of Keratinization

The goal of Dr. John DiGiovanna's research program 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. They have demonstrated that oral isotretinoin is an effective chemopreventive agent in patients with high rates of skin cancer formation. The efficacy and toxicity of isotretinoin as a chemopreventive agent is also being evaluated in a series of 8 patients with xeroderma pigmentosum and the nevoid basal cell carcinoma syndrome. A phase I/II study of intralesional recombinant human interferon gamma for basal cell carcinoma is ongoing; higher doses being used to try to improve efficacy.

Studies of DNA Repair in Normal Human Cells from Patients with Xeroderma Pigmentosum and Neurodegenerative Disorders

Dr. Jay Robbins has continued his studies of 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. Cells from patients with primary neuronal and retinal degenerations are also being studied. A collaborative project with investigators at Howard University College of Medicine has been undertaken to study DNA repair in cells from patients with Alzheimer's disease (AD). Preliminary results suggest an abnormal cytogenetic response of the AD cells which may provide the basis for a test to predict which persons at risk for AD will develop the disease and suggest that the premature death of postmitotic neurons in AD may be caused by accumulation of unrepaired DNA lesions.

METABOLISM BRANCH

The 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 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 molecular cloning of the lymphoid-restricted transcription factor, Oct-2, by Dr. Louis M. Staudt helped to define the POU-domain transcription factor multigene family. Oct-2 is expressed in B lymphocytes and is critical for the lymphoid-specific expression of immunoglobulin genes. Oct-3 functions at the earliest stages of mammalian development. It binds to the same DNA motif as Oct-2, yet has a distinct pattern of expression. Oct-3 is expressed in the pluripotent stem cells of early mammalian embryos and is then down-modulated when these cells differentiate. Oct-3 expression is, however, maintained in the germ cell lineage. The Oct-3 mRNA present in oocytes is transferred to the one-cell embryo and is absolutely required for embryonic DNA synthesis and cell division. Thus, Oct-3 is the first described mammalian maternal effect gene.

Somatic Cell Gene Therapy for Human Genetic Disease

Dr. Michael Blaese's laboratory has continued to focus on the development of gene therapy. On September 14, 1990, his group performed the first authorized use of

gene transfer to treat human genetic disease by infusing 10^9 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 deficiency patient have been treated every 5-7 weeks with such gene-corrected T-cell infusions. Each now has a normal peripheral blood T-cell count and each has begun to show signs of enhanced immune reactivity. Extensive data on the quality of immune system function and the duration of the gene transfer effect are continuing to be collected and analyzed on these patients. Similar cellular immunoreconstitution protocols are being developed to treat patients with AIDS in the coming year.

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

Dr. Jay Berzofsky has defined mechanisms by which T-cells recognize antigens presented on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and has applied these insights to the design of synthetic vaccines for AIDS, malaria, and cancer. Dr. Berzofsky has been able to quantitate the requirements for class I MHC molecules and for antigenic peptide for stimulation of CD8⁺ cytotoxic T lymphocytes (CTL). In studying epitope recognition by HIV-specific CTL, Dr. Berzofsky demonstrated that human CTL from HIV-infected individuals recognized four peptides from the envelope protein in association with HLA-A2. One of these was a dominant CTL epitope in the mouse, but three had been identified originally as helper epitopes. Conversely, he found that the CTL epitope in the mouse was also recognized by helper T cells with class II MHC molecules to helper T cells. The extent of this concordance between helper and CTL epitopes and the molecular mechanism behind it is being explored. These dual function epitopes recognized by both helper T cells and CTL may be useful components of synthetic vaccines for HIV. To overcome this problem of MHC polymorphism in the human population, Dr. Berzofsky made peptides spanning multideterminant regions of HIV gp160 and found that these were recognized by mice of multiple MHC types and by T cells from a large fraction of humans. These are being incorporated in the design of experimental vaccines.

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

Dr. Thomas Waldmann previously identified two peptides that bind IL-2: the 55 Kd protein IL2R α chain reactive with the anti-Tac monoclonal antibody, and the 70/75 Kd IL-2R β protein reactive with a monoclonal antibody termed Mik β 1. He proposed a multichain model for the high affinity receptor in which both IL-2R α - and IL-2R β -binding proteins are associated in a receptor complex. The IL-2 receptor is expressed on the abnormal cells of patients with certain forms of leukemia, autoimmune disease, and those rejecting allografts, but not on normal resting T-cells, making it an ideal target for therapy. Initially Dr. Waldmann has focused his IL-2 receptor-directed therapeutic studies on patients with HTLV-I-associated adult T-cell leukemia (ATL). The ATL leukemic cells express very large numbers of IL-2 receptors. Dr. Waldmann initiated a therapeutic trial using unmodified anti-Tac monoclonal antibody in the treatment of patients with ATL. The patients studied did not suffer any toxicity; seven of the 20 patients studied underwent at least a partial remission. However, as with all rodent antibodies, the effectiveness of anti-Tac is limited by the fact that murine monoclonal

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. 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. They have also found that the molecular basis for low TCR expression in developing CD4⁺CD8⁺ thymocytes is a high rate of degradation of newly synthesized and assembled TCR complexes, and that intrathymically generated CD4 signals regulate the TCR degradation rate in CD4⁺CD8⁺ thymocytes. Dr. Singer has examined the intrathymic differentiation of functionally and phenotypically distinct T-cell subsets, as well as their interaction with thymic epithelium. They 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.

Dr. Pierre Henkart's laboratory has collaborated with the Surgery Branch, NCI, to study human tumor-infiltrating lymphocytes (TIL) for expression of granule protein mRNA using Northern blots. They found that most TIL express mRNA for cytolysin, granzyme A, granzyme B, and serglycin and are cytotoxic when tested in "redirected" assays in which Mab against the T-cell receptor triggers the cytotoxicity. Occasional TIL are not cytotoxic and show poor expression of the cytolysin, while expressing at least some other granule genes.

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. An analysis was carried out to determine 1) the extent of T-cell receptor (TCR) V β 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. They showed that maintenance of tolerance to a variety of self-determinants results in substantial deletions in the available TCR V β repertoire. The self-determinants that function as ligands for V β -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 V β deletion, such that any one of two or more unlinked genes was permissive for deletion. Ligands responsible for deletion of V β 11- and V β 12-expressing T-cells were characterized and were shown to represent a previously uncharacterized MIs "superantigen" capable of inducing a strong response by allogeneic T-cells. Thus, the set of MIs 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.

Dr. Stephen Shaw's laboratory has been systematically analyzing heterogeneity among subsets of human T-cells and the functional capacities of those subsets. Analysis of the enormously complex phenotypes of peripheral T-cells has prompted them to propose a model of peripheral T-cell differentiation in which naive T-cells are of relatively uniform phenotype, but their activation in multiple different microenvironments gives rise to a wide variety of differentiated memory cell phenotypes.

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, it manifests an antibody-dependent cellular cytotoxicity that is absent in the parental mouse anti-Tac. Preclinical models support this predicted improved effectiveness, and a clinical therapeutic trial with "humanized" anti-Tac is being developed for use in patients with IL-2 receptor expressing malignancies. In parallel studies a "humanized" version of Mik β 1 that blocks binding to the IL-2R β component has been generated and is being evaluated for its effectiveness in blocking the interaction of IL-2 with the IL-2R β subunit.

Dr. Waldmann has developed cytotoxic agents wherein α - and β -emitting radionuclides are conjugated to anti-Tac by use of bifunctional chelates. The β -emitting radionuclide yttrium-90 has been conjugated to anti-Tac using chelates that neither damage the antibody nor permit the elution of radiolabeled yttrium from it. In a dose escalation trial using yttrium-labeled anti-Tac to treat HTLV-I-associated adult T-cell leukemia (ATL), no toxicity was observed in 5 of 6 patients studied, and five of the six patients underwent a sustained, partial or complete remission. Thus it is hoped that yttrium-90 chelated to "humanized" Mik β 1 will prove to be an effective, relatively nontoxic agent for the treatment of an array of human leukemias.

Biology of the Immune Response

Dr. David Nelson identified a soluble form of the IL-2R α component (Tac protein) of the human IL-2 receptor in the culture supernatants of activated T cells, B cells, and monocytes *in vitro* and in the sera of normal individuals *in vivo*. Elevated levels of soluble IL-2R α have been demonstrated in the sera of patients with hairy cell leukemia (HCL), human retroviral diseases including adult T-cell leukemia (ATL) and the acquired immune deficiency syndrome (AIDS). In ATL and HCL patients the serum level IL-2R α was indicative of tumor burden, and favorable responses to therapy were associated with reductions in the serum level of IL-2R α . Elevations of IL-2R α in the serum were also indicative of allograft rejection episodes in patients receiving liver and heart-lung allografts. Patients with autoimmune diseases also had elevated levels of IL-2R α in the serum and joint fluids. The measurement of soluble IL-2R α in various body fluids is thus useful in monitoring certain neoplastic and immune-mediated events *in vivo*.

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; transplantation biology; tumor immunology; and flow cytometry.

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Studies of T lymphocyte responses in HIV⁺, asymptomatic individuals by Dr. Gene Shearer's laboratory indicate that 75-80% of these patients exhibit selective CD4⁺ T-helper cell (Th) functional defects without any AIDS symptoms and without loss of CD4⁺ cell numbers. A defect of antigen-presenting cell (APC) function was not observed in any such asymptomatic individuals, although two distinct types of APC defects were detected in AIDS patients. The defect in CD4⁺ Th function was associated with suppressor T-cells that produced a soluble factor that was also capable of selectively suppressing Th function. Dr. Shearer's laboratory has developed a very sensitive Th assay that permits the detection of HIV exposure and/or infection three to fourteen months before detection by the antibody or polymerase chain reaction assays. This sensitive assay has also been used to detect subtle immunologic changes induced by anti-viral drug therapy, and HIV-specific, Th immunization in volunteers immunized with a trial AIDS vaccine. Dr. Shearer's laboratory has also investigated cell-mediated immune function defects in patients with the Wiskott-Aldrich (W-A) primary immune deficiency, and has observed a series of T lymphocyte and antigen-presenting cell defects similar to those found in HIV-infected individuals.

Cell Biology of Immune Responses

The expression and function of cell adhesion molecules by B-cells was analyzed by Dr. Richard Hodes' laboratory. Their studies suggest that CD44 expressed on activated B-cells may serve as an adhesion molecule playing a role in B-cell trafficking in vivo, and its adhesive function may be regulated at the level of quantitative expression, as well as qualitative differences in the expressed molecules.

Dr. Shaw's laboratory has been identifying and characterizing the functions of cell surface molecules which facilitate T-cell recognition. Particular progress has been made in understanding the molecular basis of T-cell interactions with endothelium, which is critical to T-cell recirculation and migration. Two new molecular pathways are being elucidated. Dr. Shaw's group has also defined many characteristics of the molecule CD31 on a subset of T-cells which make it a very attractive candidate for regulating T-cell adhesion to endothelium, and suggest that it is a critical element in a T-cell adhesion cascade. T-cell interaction with endothelium via three other molecular pathways, VLA-4/VCAM-1, LFA-1/ICAM-1, and LFA-1/ICAM-2, has also been systematically analyzed. The relative importance of these multiple pathways depends critically on the state of activation of the T-cell and of the endothelium, as well as on the subset of T-cells. Studies from Dr. Shaw's laboratory have highlighted the importance of regulation of adhesion to understanding T-cell function.

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. It was shown that the signalling pathways utilized by cloned T-cells were influenced by prior stimulation through the TCR.

Dr. Allan Weissman's laboratory has been interested in the T-cell antigen receptor zeta subunit, which is a key structure in receptor-mediated signal transduction. The zeta subunit is a substrate for a receptor-activated protein tyrosine kinase and undergoes multiple, apparently cooperative, phosphorylations

in response to receptor activation. In order to determine which tyrosines of zeta are phosphorylated and to understand the functional consequences of these modifications, a systematic mutational analysis of the zeta subunit has been initiated. Future studies will assess the role of phospho-zeta by the generation of transgenic animals containing the mutation at residue 111. Dr. Weissman's group has developed a permeabilized cell system for the study of T-cell hybridomas. This will enable them to study the molecular mechanism responsible for coupling receptor occupancy to signalling pathways.

Structure, Regulation and Function of Genes Involved in Immune Responses

The MCH class I molecules, which serve as the targets of cellular immune responses and allograft rejection, are expressed on nearly all somatic tissues. In order to investigate the molecular basis for the differential patterns of expression, the laboratory of Dr. Dinah Singer has begun to identify and analyze regulatory DNA sequence elements and to generate a series of transgenic mice containing variants of the class I regulatory domain. Her studies suggest that class I genes are negatively regulated and that tissue-specific levels of gene expression result from an equilibrium between the activities of the negative and positive elements associated with the complex.

In addition to tissue-specific regulation, MHC class I gene expression is known to be affected by immunomodulators, which can either increase or decrease levels of expression. Agents such as tumor necrosis factor and interferon are well known modulators of class I genes. Dr. Singer's laboratory has recently observed that the thyroid-stimulating hormone specifically reduces transcription of endogenous class I genes in cultured thymocytes. cAMP can reduce class I gene expression in thymocytes and in a variety of cells including thymocytes, lymphoblasted cells and fibroblasts. Interestingly, c-jun is also a negative regulator of class I expression.

Dr. Singer's laboratory has identified a new MHC subregion, M, which contains a set of class I genes which are highly divergent from other members of the MHC family. The three most closely related members of the M family have been isolated and sequenced. All three are capable of encoding protein products, although no transcription has yet been observed. Transgenic mice have been generated in which the expression of one of the genes, M1, is directed by a viral LTR promoter. Analysis of the expression of M1 in these lines is in progress.

Lymphocyte Effector Function

Dr. Pierre Henkart's laboratory has sought to test the granule exocytosis model for lymphocyte cytotoxicity by examination of the cytotoxic activity of a rat mucosal mast cell tumor line (RBL) transfected with genes for cytotoxic lymphocyte granule components. RBL, which degranulates in response to cross-linking its IgG Fc receptor, is not cytotoxic, but acquires a potent lytic activity against IgE-coated red cells when transfected with the mouse cytolytic (cy) gene. Preliminary results add support to the hypothesis that granzyme A triggers target DNA breakdown after gaining access to the target cell cytoplasm.

Using a murine model system developed in his laboratory, Dr. Ronald Gress has shown that the administration of anti-CD3 monoclonal antibody in vivo can suppress cytotoxic T-cell function and enhance engraftment, and that this is due to both suppression of host T-cell function and the presence of growth factors.

Dr. Gress' laboratory has developed approaches for depleting normal and malignant T-cell marrow populations as a means of assessing the feasibility of utilizing allogeneic HLA-mismatched, T-cell-depleted allogeneic marrow and autologous marrow purged of malignant T-cells in the treatment of aggressive hematolymphopoietic malignancies. Preclinical studies demonstrated that functional T-cell populations are generated in animals receiving T-cell-depleted autologous marrow. The functional capacities of regenerated T-cell populations following T-cell-depleted marrow transplantation are also of interest. The human T-helper cell response to xenogeneic MHC encoded antigens expressed by stimulating murine cell populations has been studied and found to be of special use in the assessment of human T-helper cell function.

Tumor Immunology

Bispecific antibodies, with specificities for triggering structures on cytotoxic cells and for cell-surface structures on target cells, redirect the target-cell specificities of cytotoxic cells. Drs. David Segal and John Wunderlich have demonstrated that targeted human peripheral blood lymphocytes block the growth of human ovarian carcinoma cells established in the peritoneal cavity of immunodeficient mice. Treating the 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. This provides an animal model for using targeted T-cells to treat ovarian cancer; clinical trials in women with ovarian cancer are now commencing in The Netherlands and in Italy.

Drs. Segal and Wunderlich have also initiated studies of targeted cytotoxicity in vivo, using a fully syngeneic mouse model consisting of mouse mammary tumors induced by the mammary tumor virus (MTV). A monoclonal antibody (P2A112) binds to the surfaces of spontaneous mammary tumors and to cultured mammary tumor lines. When cross-linked to an antimurine CD3 MAb, the P2AE12 x anti-CD3 bispecific antibody induced murine T-cells to lyse mammary tumor lines and spontaneous mammary tumors and blocked the growth of mammary tumor cells in culture. The mouse mammary tumor model, therefore, appears promising as a totally syngeneic murine system for studying immune targeting with bispecific antibodies in vivo.

IMMUNOLOGY BRANCH

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

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

The induction of intentional mixed chimerism across major histocompatibility complex (MHC) barriers has been investigated as a means of producing specific transplant tolerance. The use of mixtures of T-cell depleted syngeneic plus allogeneic bone marrow has been found to produce permanent, specific tolerance with full immunocompetence. This model has been found to have potential

usefulness in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect.

Dr. Sachs and his colleagues have shown that treatment with monoclonal anti-T-cell subset antibodies plus selective thymic irradiation can produce long-term mixed chimerism and specific tolerance across an MHC barrier with a non-lethal preparative regimen. This regimen has now been successfully applied to rat-mouse xenogeneic chimeras as well. Recent studies have demonstrated the presence of natural antibodies in mouse serum reactive with rat bone marrow cells that seem to correlate with graft rejection. These results may explain the requirement for much higher numbers of rat bone marrow cells than allogeneic bone marrow cells to obtain engraftment.

Miniature Swine Model

Partially inbred strains of miniature swine, NIH mini-pigs, have been developed as a large animal preclinical model for transplantation studies. This model has made it possible to determine that class II antigen matching is of overwhelming importance in determining the outcome of vascular allografts such as kidney transplants. The molecular analysis of class II genes is in progress.

Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference have been found to frequently develop specific systemic tolerance involving a specific depletion of class I reactive helper T-cell populations. Also consistent with lack of T-cell help, recipients of class II-matched renal allografts which went on to accept such grafts long-term showed a brief rejection crisis at approximately 14 days. An ablative radiation regimen has been established in the miniature swine as a model for HLA identical sibling transplants in man, and has been found satisfactory to permit MHC-matched bone marrow allografts.

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY

A major project within the Laboratory of Tumor Immunology and Biology, directed by Dr. Jeffrey Schlom, is the development of monoclonal antibodies directed against tumor-associated antigens for the diagnosis and treatment of cancer. Mab B72.3, a murine IgG1, has been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, with minimal reactivity to normal adult tissues. Mab B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving over 1000 patients. A series of "second generation" Mabs to the TAG-72 antigen have been characterized, i.e., CC83 and CC49. A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain and light chain constant regions.

Tumor targeting and pharmacokinetic studies were also carried out using a genetically engineered single chain antigen-binding protein (sFv). These studies demonstrated that a relatively small (27kD) single chain molecule can efficiently target a human tumor xenograft.

Anticarcinoma Monoclonal Antibody Clinical Trials

The selective localization of ¹³¹I-Mab B72.3 IgG was demonstrated in a study of colorectal cancer patients. Dr. Schlom and his colleagues 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 intraperitoneal administration of ¹³¹I-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity is in progress. The use of recombinant-chimeric Mabs has also begun, and the use of second and third generation Mab-isotope conjugates are planned.

Active Immunotherapy to Human Carcinoma-Associated Antigens

Carcinoembryonic antigen (CEA) is a 180,000 dalton glycoprotein expressed on most gastrointestinal carcinomas and many other human carcinoma types. Dr. Judy Kantor in Dr. Schlom's group has isolated a 2.4-kb cDNA clone containing the complete coding sequence from a human colon tumor cell library and inserted it into a vaccinia virus genome. This newly developed construct was characterized by Southern blotting, DNA hybridization studies, and polymerase chain reaction analysis. The CEA gene was stably integrated in the vaccinia virus thymidine kinase gene. The recombinant was efficiently replicated upon serial passages in cell cultures and in animals. The recombinant virus expresses, on the surface of infected cells, a protein product recognized by a monoclonal antibody directed against CEA. Immunization of mice with the vaccinia construct was found to elicit a humoral immune response against CEA. Pilot studies also demonstrated that the administration of the recombinant CEA vaccinia construct was able to greatly reduce the growth in mice of a syngenic murine colon adenocarcinoma which had been transduced with the human CEA gene. The use of this new recombinant CEA vaccinia construct may provide an approach in the specific active immunotherapy of human gastrointestinal cancer and other CEA-expressing carcinoma types including some breast cancers.

Mammary Tumorigenesis in Inbred and Feral Mice

MMTV appears to induce mammary tumors in mice by acting as an insertional mutagen that leads to the activation of a previously silent gene or the rearrangement of a normally expressed gene (*int* genes). Several viral-induced preneoplastic hyperplastic outgrowth (HOG) lines have been developed, three of which have a viral insertion at *int-1*. A new *int* locus, designated *int-6*, has been found in a fourth HOG line. Many of the HOG lines spontaneously give rise to mammary tumors, some of which metastasize. Both the primary tumors and the metastatic lesions frequently contain additional viral insertions over those observed in the particular HOG line. This suggests that it may be possible to identify new *int* genes that are associated with the particular stages of malignant progression. Other studies have shown that the *int-2* gene product can functionally replace bFGF and compete with it for cellular receptors.

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. Dr. Robert Callahan's group has undertaken a program that is aimed at determining the molecular genetic alterations in primary breast tumor DNA that have a statistically significant association with prognosis. In previous studies, they detected frequent amplification of the *c-myc*, *int-2*, and *c-erbB2* proto-oncogenes and the frequent loss of heterozygosity (LOH) on chromosomes 1q, 3p, 11p and 13q. Current results demonstrate that LOH also frequently occurs on chromosomes 1p, 17p, 17q, and 18q. LOH on chromosome 17q has a significant association ($p < 0.02$) with estrogen receptor negative tumors, and LOH is associated ($p < 0.04$) with histopathological grade III tumors. They have also found associations between specific mutations, one subset of tumors could be defined by the frequent presence of LOH on chromosomes 11p, 17p, and 18q, another subset of tumors contained LOH on chromosomes 1p, 13q, and 17q. These results suggest that different subsets of mutations, possibly acting in a complimentary way, are a consequence of the heterogeneous nature of the etiologic factors that provide the selective pressure for the clonal outgrowth of cells containing particular mutations during breast carcinogenesis. Studies are in progress to identify the target genes affected by LOH. The p53 gene on 17p is a likely candidate. A LOH has been found at the NM23 gene on 17q. This gene appears to be tightly linked to the hereditary breast cancer locus.

SUMMARY REPORT

LABORATORY OF GENETICS, DCBDC, NCI

October 1, 1990 through September 30, 1991

The investigators in the Laboratory of Genetics carry out a diverse set of investigations, many of which focus on genetic changes that take place in the neoplastic transformation of hematopoietic cells. These include susceptibility and resistance genes that predispose to tumor formation, oncogenes and genes that influence tumor development. One model system studied by several members of the Laboratory of Genetics is the paraffin-oil induced plasmacytomas in mice. The genetic constitutions play a role in the development of these tumors, and some of these genes are sensitive to environmental influences. BALB/cAn mice are highly susceptible to developing plasmacytomas while most other strains, e.g., DBA/2, are resistant. One of the major projects is to identify and characterize the several genes that determine susceptibility and resistance. Some of the genes of DBA/2 origin that determine partial resistance to plasmacytomagenesis are located on a 30 cM segment on chromosome 4 between the IB-4 and Anf loci. BALB/c.DBA/2 congenic strains carrying portions of this segment of chromosome 4 have been developed. These congenic mice develop a much reduced incidence of plasmacytomas and far fewer plasma cell proliferative lesions (foci) during the latent period (days 120-150). The reduced formation of foci provides a phenotype for determining susceptibility and resistance more rapidly than by tumor induction. We are searching for correlative phenotypes and have evidence that both DBA/2, BALB/c.DBA/2-chr 4 congenics Fr-1^{N/N} N19; Tol fam-3, efficiently repair UV induced DNA damage to the 5' end of the c-myc oncogene more efficiently than BALB/cAn mice. We are attempting to localize Pctr genes on chromosome 4.

Dr. Beverly Mock has injected 750 BALB/c x (BALB/c x DBA/2)F1 1st generation backcross mice with pristane and induced the expected 11-13% plasmacytomas. She has genotyped these tumors for a variety of polymorphic genes and found linkage of susceptibility to the chromosome 4 markers Ls1, Ifa, D4Lgm3, Sc1, D4Rpl and D4Lgm1.

Plasmacytomagenesis in BALB/c mice is influenced by environmental factors. Linda Byrd (graduate student) has shown that specific pathogen-free BALB/c mice are refractory to plasmacytoma induction.

Dr. Emily Shacter has been investigating the role of IL-6 in the oil granulomas induced by pristane of BALB/c and DBA/2 and has found differences in production of IL-6 by peritoneal exudate cells. Cells from BALB/c mice produce more IL-6.

The deregulation of the c-myc oncogene is a critical event in plasmacytomagenesis that is caused by chromosomal translocation. The molecular mechanisms of deregulation are not yet understood. Some of these myc-activating translocations occur 200-300 kb 3' of c-myc in a region called Pvt-1, and Drs. Mushinski and Huppi have shown that this locus is transcribed at very low levels in normal cells but in much higher amounts in some plasma-

cytomas. Normally Pvt-1 transcripts are ca. 14 kb in length, suggesting that the Pvt-1 locus is very large. In some plasmacytomas with chromosomal translocations in the Pvt-1 locus, the Pvt-1 transcripts are truncated and form chimeras with Ig kappa chain transcripts. Drs. Huppi and Mushinski are attempting to define the Pvt-1 locus and have identified new, distant exons. The function of Pvt-1 remains obscure.

Dr. Mushinski has isolated a cDNA for mouse bcl-3 (on human chromosome 19) and have mapped it to mouse chromosome 7. During B-cell differentiation its expression is maximal in cells that have not undergone Ig isotype switch. This suggests that the chromosome translocations involving this locus that characterize some CLL's occur in this stage.

The ABL-MYC retrovirus, expressing v-abl and c-myc, rapidly induces intraperitoneal plasmacytomas in BALB/c and other strains of mice, even in the absence of i.p. oil and in the absence of helper virus. If the mice are preimmunized with lysozyme or sheep red blood cells, about 60% develop tumors producing antibody specifically directed against the immunogen. This may prove to be a useful alternative to hybridoma technology for generating monoclonal antibodies.

Dr. Linda Wolff is continuing to study the development of acute monoclonal leukemias in mice that are induced by the intravenous injection of Moloney Leukemia Virus in pristane conditioned mice. In a very high percentage of these tumors MoMuLV inserts into the myb oncogene. Dr. Wolff has used PCR to detect Moloney virus-myb junction gene and can detect these translocations at the molecular level before the tumors appear. This step in the leukemogenic process can be detected as an early step and may represent a pre-leukemic mutation. Pre-leukemic cells can be detected in hematopoietic organs such as bone marrow, liver, spleen and mesenteric granuloma as early as 3 wks post viral infection even though the disease is not evident until 3-4 mo. Although pristane increases the number of mice in which such cells can be detected, they can be detected even in the tissues of some mice that have not been treated with pristane. Future experiments will be aimed at extending our knowledge about trafficking and the role of hematopoietic organs in this disease.

Dr. Rudikoff has been studying the biological basis for DBA/2 resistance to pristane-induced plasmacytomagenesis by utilizing a series of transfer experiments introducing either BALB/c or DBA/2 bone marrow into SCID mice followed by tumor induction protocols. Tumor induction has employed either classic pristane treatment or the use of a retroviral construct, J3V1, supplying deregulated myc and raf oncogenes. These studies indicate that SCID mice reconstituted with BALB/c bone marrow and injected with J3V1 develop plasmacytomas and myeloid tumors. In contrast, DBA/2 reconstituted SCIDs develop only myeloid tumors indicating that the genetic difference between the two strains, in terms of plasmacytoma induction, resides in the DBA/2 B-cell and that resistance cannot be overcome by the introduction of a deregulated myc oncogene which seems to be critical for induction in BALB/c.

Dr. Wendy Davidson has studied CD45 isoform expression during B lymphocyte and myeloid differentiation, and the effects of the mutant genes lpr and gld on T cell development and function. By PCR analysis and immunoprecipita-

tion, she has shown that the 220 kDa isoform of CD45 is expressed on B lineage cells from pro-B cells to B immunoblasts. Differentiation into plasma cells was accompanied by a switch to two lower MW isoforms, a predominant species that spliced out exons 4, 5 and 6 and a minor species that spliced out two of the three exons. By comparison, myeloid cells spliced out exons 4, 5 and 6 early in development and retained this phenotype in mature cells. Dr. Davidson has continued to explore basic mechanisms in the pathogenesis of lymphoproliferative diseases in mice carrying the lpr and gld mutations. She has shown that a high proportion of CD4⁺ and CD8⁺ T cells expressed high levels of CD44 and reduced levels of Mel 14 and CD45R, a phenotype consistent with T cell activation. These two subsets also hypersecreted IL-2, IFN- γ and TNF following stimulation. By comparison, the two unique B220⁺ T cell subsets had the phenotype of less mature T cells were refractory to stimulation and did not secrete cytokines.

The X-ray structure of a third HyHEL-26 Fab-HEL complex has been solved and is being refined. The HyHEL-26 epitope is nearly identical to that recognized by HyHEL-10, and the primary and genetic structures of HyHEL-26 are closely related to those of HyHEL-8 and -10. These results represent the first time structures have been obtained for 2 structurally related antibodies complexed to the same protein epitope, and we now have an unprecedented system for studying structure-function relationships. Dr. Smith-Gill has begun to experimentally investigate structure-function relationships in the interface by site-specific mutagenesis of both antigen and antibodies. These studies are beginning to define fundamental principles that will allow prediction of function from structure, principles that are critical to such applications as anti-protein antibody design and vaccine development. Dr. Smith-Gill is also approaching the problem of vaccine development by investigating immunogenicity and protective epitopes in *Shigella flexii*.

Dr. Hayden Coon continues to develop new and difficult systems for cell culture. He has been 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. He has 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.

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

PROJECT NUMBER

Z01 CB 05552-22 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mammalian cellular genetics and cell culture

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

P. I.:	H.G. Coon	Research Biologist	LGN, NCI
	A. Degrassi	Visiting Scientist	LGN, NCI
	B. Wolozin	Staff Fellow	NIMH, LCS

COOPERATING UNITS (if any)

Prof. P. Graziadei, Dr. A. Monti-Graziadei, Florida State Univ., Tallahassee, FL; Prof. F. S. Ambesi-Impombato, 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 MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0.0

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

B

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

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:

During this year real progress has been made in spite of the lack of technical help in the first third of the year. This progress, however, will generally not be understood, nor will it be of much interest to the various bureaucrats who will read this document. Their lack of understanding and imagination has led to severe funding restrictions that have prevented any quantitation in our work so that their lack of appreciation becomes a self-fulfilling prophecy. Nevertheless, I shall try briefly to list the real high points in the forlorn hope that history will one day vindicate my position.

Human olfactory neuroblasts:

During this year the NIMH has gotten the protocol approved for doing biopsies of human olfactory epithelium from some Alzheimer's and from some controls. We have found that the control biopsies could be grown up into full fledged olfactory neuroblast populations as good as those we had been getting at autopsy. Furthermore, my collaborator, Dr. Ben Wolozin, has found that the cells from either source can be gotten to differentiate to the point of accumulating the olfactory sensory neuron marker: olfactory marker protein. When the cultures are grown in the presence of TPA, NGF, and Ca^{++} , they will start accumulating OMP and show good bands on Western blots. These are very interesting points and they should be followed up. Are these the same neuroblasts that have been synthesizing neurofilaments? Have those members of a culture that are OMP synthesizers also neurofilament synthesizers? In the past year it has been twice confirmed that there was no detectable neurofilament protein to be found by immunohistochemistry in the olfactory epithelium itself.

Without the ability to look for furaII- Ca^{++} stimulation there is no chance that our lab will be involved with any further advances in understanding the olfactory system neurons. It is also apparent that there will not be enough money in the present budget to support the grafting experiments discussed in last year's report. These grafting experiments involve extensions of work I have done in the past: the grafting of retrovirally labelled with bacterial beta-galactosidase tissue culture cells by implanting a pseudotissue into various host sites and host physiological conditions. There is reason to believe that it is important to do this with the human olfactory neurons since they may be making a sort of "genetic" neuron in culture that might differentiate in different and useful pathways in CNS sites other than the OE. This work, of course, has immensely important implications in medicine and in the treatment of many neurological disorders for which fetal tissue transplants now seem to be the only choice. At all events, it appears that the olfactory neuroblast project will have to be wound down and eliminated in the coming year for lack of support.

Improved primary cultures of human normal and tumor cells:

As noted in last year's annual report our media developed for the olfactory neuroblasts have been evolved into media that provide sometimes spectacular improvement in the ability to grow primary human cells. This was first noted on cultures of human thyroid. For some 15 years we had tried off and on to grow human thyroid cells as well as we had been able to grow rat

thyroid cells (FRTL and FRTL-5). This year I have found the human thyroid cells found to be exquisitely sensitive to TSH. Perhaps the main reason no one has succeeded in culturing human thyroid cells is that these cultures have always been hundreds of times over-stimulated with TSH. We now have a human cell culture system that is substantially better than the already very useful rat system. Various human diagnostic procedures are expected to improve from the availability of human thyroid cell strains.

These thyroid cell strains have been made successfully from glands in various thyroid disease states including: goiter, adenoma, carcinoma, as well as from normal tissue. One interesting feature of these cultures is that virtually every cell in the follicle appears to participate in the divisions in culture. This has not always been the case: often a particular cell type or a cell in a particular state has grown out to the exclusion of others. Of course, after a relatively small number of divisions, the population will be overwhelmingly composed of the faster growing cell types. Short of primary or early cloning, there is nothing that can be done about this problem. The observation, however, suggested that we try various tumor biopsies, where cellular heterogeneity has often caused difficulty in assessing tumors in culture (e.g., for chemotherapy screening). Our small survey of human primary tumors has tended to bear out this finding. We have tested 3 human breast cancers, 1 cervical cancer, a colon cancer, a hepatoma, and various thyroid cells. All seem to grow very well in our media. The media have been tested against standard test sarcoma cell lines by a person in the chemotherapy testing business (Boris Rotman, Brown University, Providence, RI) and found to yield anywhere from 20 to 40 times better performance (greater ^3H -thymidine incorporation) than do standard culture media (199 and RPMI 1640 were tested). Surely it is ironic that the National Cancer Institute is unwilling to rally around and to make a sincere effort to put this kind of discovery on a sound statistical footing.

Perhaps the most important discovery we have made in the present year has been the finding that cells from the glandular pancreas can now be grown in culture. If we can bring this to an obvious conclusion it will truly revolutionize an important branch of medical therapy. I intend to try, whether the bureaucrats like it or not.

Publications:

DeGrassi A, Hilbert DM, Anderson AO, Potter M, Coon HG. In vitro culture of a primary plasmacytoma that has retained its dependence on pristane conditioned microenvironment for growth. *Curr Top Microbiol Immunol* 1990;166: 71-6.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05553-22 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Immunoglobulin structure and diversity. Characterization of cell membrane proteins

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

P.I.: Stuart Rudikoff	Microbiologist	LGN, NCI
J. Hanley-Hyde	Staff Fellow	LGN, NCI
D. Hilbert	Staff Fellow	LGN, NCI
P. Hausner	Visiting Fellow	LGN, NCI

COOPERATING UNITS (if any)

Dr. K. Holmes, NIAID, NIH; Dr. A. Anderson, US Army Institute of Infectious Diseases, Frederick, MD

LAB/BRANCH

Laboratory of Genetics

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Molecular Immunology Section

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TOTAL MAN-YEARS:

6

PROFESSIONAL:

4

OTHER:

2

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

B

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

I. Questions related to abnormal lymphocyte development are being approached in the plasmacytoma model developed by Dr. Michael Potter. One of the basic observations of plasmacytoma induction is that the BALB/c strain is highly susceptible, but others, including DBA/2, are resistant. We have examined the biological basis for DBA/2 resistance by a series of transfer experiments introducing either BALB/c or DBA/2 bone marrow into SCID mice followed by tumor induction protocols. Tumor induction has employed either classic pristane treatment or the use of a retroviral construct, J3V1, supplying deregulated myc and raf oncogenes. These studies indicate that SCID mice reconstituted with BALB/c bone marrow and injected with J3V1 develop plasmacytomas and myeloid tumors. In contrast, DBA/2 reconstituted SCIDs develop only myeloid tumors indicating that the genetic difference between the two strains, in terms of plasmacytoma induction, resides in the DBA/2 B-cell and that resistance cannot be overcome by the introduction of a deregulated myc oncogene which seems to be critical for induction in BALB/c. II. The SCID mouse has additionally been used to study normal lymphocyte differentiation. We have initially reconstituted SCIDs with Peyer's patch cells as representatives of populations found in a specialized component of the mucosal immune system. Reconstituted SCID mice have been analyzed over a period of 6 months to establish both serum and tissue profiles. Reconstituted mice display a serum immunoglobulin profile similar to normal animals both qualitatively and quantitatively in spite of the fact that Peyer's patch B cells express only IgM and IgA. All lymphoid tissues including mucosal surfaces (gut, lungs), peripheral organs (spleen, lymph nodes) and the thymus are reconstituted. Normal anatomical structures such as spleen and lymph node germinal centers are readily visible in tissue sections. Thymic tissue reveals normal anatomical areas (cortex and medulla), but repopulation is by mature, single positive (CD4) or (CD8) T cells. Results of these studies have important implications for models of organ specific lymphocyte homing as well as concepts of cellular commitment during differentiation.

Major findings:

1. Plasmacytomas arise in granulomatous tissue formed following the intra-peritoneal injection of mineral oils or pristane. A number of genetic analyses have established susceptibility and resistance patterns for plasmacytoma induction among a variety of inbred mouse strains. These phenotypes appear to be complex and associate with multiple genes located on different chromosomes. While studies are underway in the Laboratory of Genetics to identify genetic loci encoding these traits, little is known about the biological level at which these phenotypes are expressed.

For plasmacytoma induction, the BALB/cAN strain is the prototype for susceptibility, while DBA/2 is resistant. A basic question to be addressed in this process concerns the level at which DBA resistance is manifest. The resistance could be inherent in the DBA B-cell, in an accessory cell or the ability to form an appropriate granuloma capable of supporting neoplastic development. Since the granuloma induced in the DBA strain is qualitatively different from that found in BALB/c, it has previously not been possible to assess the contribution of B versus accessory cells to resistance. In order to unambiguously approach this question, SCID mice have been reconstituted with bone marrow from tumor susceptible (BALB/c) and resistant (DBA/2) strains. Bone marrow from either BALB/c or DBA/2 leads to successful reconstitution of all lymphoid tissues as assayed by Fluorescence Activated Cell Sorter (FACS) or appropriate staining of frozen tissue sections.

Reconstituted SCID mice have been subjected to tumor induction protocols involving injection with pristane and a retrovirus containing myc and raf oncogenes, J3VI, or pristane alone. Animals receiving BALB/c bone marrow followed by J3VI and pristane develop both plasmacytomas and myeloid tumors. A tumor incidence of 68% was observed with 25% plasmacytomas and 38% myeloid tumors as determined by FACS analysis and immunoglobulin secretion. In the DBA reconstituted animals only myeloid tumors were observed with an incidence of 41%. Experimental groups receiving pristane alone have failed to develop tumors after a period of one year.

Results of these experiments indicate that SCID mice reconstituted with BALB/c bone marrow develop both lymphoid and myeloid tumors of donor origin whereas DBA reconstituted animals develop only myeloid tumors. Since both susceptible and resistant cells are in the context of the same permissive granuloma, plasmacytoma resistance must be a reflection of properties inherent to the DBA B-cell. It is interesting to note that the myeloid compartments of both strains are susceptible to tumor induction and it is only in the DBA B-cell compartment that resistance is observed. Furthermore, de-regulation of the myc oncogene by chromosomal translocation has been implicated as a critical event in plasmacytoma induction as well as possibly Burkitt's lymphoma. DBA reconstituted animals given J3VI are, in effect, receiving a de-regulated myc gene yet do not develop plasmacytomas. This observation suggests that a possible inability of DBA B-cells to undergo chromosomal translocations resulting in myc de-regulation is not sufficient to explain DBA resistance. Thus, other, as yet uncharacterized events, are critical to this tumorigenic process and/or the complex effect of background genes in a particular strain may be a major determining factor in susceptibility.

2. Lymphocyte development is an exceedingly complex process involving multiple cell-cell interactions as well as responses to a large spectrum of soluble factors secreted both by lymphocytes and other cell types. Furthermore, lymphocytes do not develop statically but circulate throughout the body at extremely high rates. During the last several years, the contributions of many laboratories have begun to unravel some of the basic mechanisms involved in lymphocyte development.

This laboratory has become interested in lymphocyte developmental aspects associated with circulation and tissue population. As such, the concepts of organ-specific lymphocyte homing and molecules associated with this phenomenon are of particular importance. Several lines of investigation have suggested that lymphocytes express specific receptors that lead to migration through high endothelial venules into lymph nodes. Among these receptors are CD44, LFA-1, the integrin VLA-4 and MEL-14. MEL-14 has further been suggested to function as an organ-specific receptor involved in the localization of lymphocytes to peripheral lymph nodes whereas VLA-4 is involved in homing to Peyer's patches. The concept of organ-specific homing is intriguing, but the regulation of such a process at the cellular level would appear problematic in terms of the need to have specific lymphocyte populations in a given organ site at a particular time. It is therefore important to experimentally determine the ability of lymphocytes from a particular source to populate other lymphoid tissues.

To address this question, we have again elected to take advantage of the SCID mouse as an experimental model. Studies currently in progress involve the reconstitution of these animals with Peyer's patch cells as a source of lymphocytes from a specialized organ of the mucosal immune system. The donor strain selected is DBA/2 which has no major histocompatibility differences from the recipient SCID (BALB/c) but does express a serological lymphocyte marker (Qa-2) absent in the SCID permitting unambiguous identification of donor cells. Peyer's patch cells have been i.v. injected into SCID recipients 4-6 weeks old and reconstitution determined at various time points by serum, FACS and histological analysis.

The Peyer's patch donor cells contain approximately 65-75% B-cells and 20% T-cells. Eighty to 90% of the B-cells express IgM and 10% IgA. The T-cells predominantly express the CD4 marker characteristic of 'helpers' with only 2-5% CD8 positive (cytotoxic). None of the T-cell population express both CD4 and CD8 and all are positive for the alpha,beta form of the T-cell antigen receptor indicating mature, peripheral T-cells. Serum analysis reveals that by 8 weeks reconstituted animals express normal or slightly elevated levels of IgM, IgA and, importantly, IgG. The levels of IgG observed provide experimental evidence that Peyer's patch B-cells can give rise to progeny producing a normal immunoglobulin spectrum and raise questions concerning the suggested pre-commitment of these cells to IgA production.

FACS analysis of spleens from reconstituted animals reveals that by day 3 about 6% of the organ is composed of T-cells (50% CD4+ and 50% CD8+) and 4% B-cells. By 5 weeks, and until 6 months, T-cells comprise 10-15% of the spleen and represent more than 200% of the total T-cells injected. The ratio of CD4+/CD8+ cells approximates that of the donor population (6:1). B-cell levels

appear to increase steadily and by 5 months represent 25% of the total spleen. At each time point virtually 100% of the CD4+ or CD8+ T-cells, as well as the IgM+ B-cells express the Qa donor marker. Histological examination reveals that the repopulating cells are not randomly dispersed throughout the spleen, but form normal structures. For example, typical germinal centers are seen which, by immunochemical staining, have expected, well defined B and T-cell zones. The histological analysis further reveals that all lymphoid tissues are reconstituted including mucosal elements such as the lung and lamina propria of the gut, peripheral elements including the spleen and other lymph nodes and, most surprisingly, the thymus. In all tissues both B and T-cells are actively dividing and, with the possible exception of the thymus, appear to form normal structures. Additionally, a comparison of spleen versus lymph node suggests a higher order degree of reconstitution specificity in that the ratio of T:B cells in the spleen is approximately 1:1 whereas in pooled lymph nodes the ratio is approximately 10:1 while that of the donor population is 1:4.

Thymic reconstitution was unexpected in these studies as the donor Peyer's patch population contains only mature, single positive (CD4 or CD8) cells. Although some CD4+ cells are found in normal thymus, it is not clear that these are mature T-cells as opposed to immature cells which will soon exit. Mature CD8+ T-cells are not believed to return to the thymus. FACS analysis of pooled thymus tissue reveals the presence of 14% CD4+ and 15% CD8+ cells and no cells expressing both markers (double positive). While the possibility may be raised that the tissue examined is contaminated with parathyroid lymph nodes, this seems unlikely as there are no B-cells present. Furthermore, serial sections clearly reveal the presence of non-overlapping sets of CD4+ and CD8+ cells in thymic tissue. The absence of double positive cells (CD4+,CD8+), which are precursors of the single positives, indicates that the thymus has been repopulated by mature, single positive peripheral T-cells. These results suggest that, at least in the absence of normal thymic components as represented in the SCID mouse, peripheral T-cells can return to the thymus raising the possibility that similar cell trafficking may occur in 'normal' development.

The above described experiments demonstrate that lymphocytes from a specialized element of the mucosal immune system are capable of repopulating all lymphoid tissues. This observation places a number of constraints on hypotheses of organ specific lymphocyte homing. If the assumption is made that all cells entering the Peyer's patches express receptors specific for mucosal tissues, then a significant proportion of these cells must be capable of modulating receptors to create specificities for peripheral tissues. It is of further interest that one putative homing receptor, MEL-14, which was originally suggested to be involved in homing to peripheral tissue, is found on greater than 50% of Peyer's patch lymphocytes. Therefore, either MEL-14+ cells are capable of entering mucosal tissues or such molecules can be induced in these tissues to generate cells with new homing specificities. If lymphocytes do home in an organ specific fashion, it appears as though significant fluidity would be required to explain the present results.

Publications:

Frels WI, Bordallo C, Golding H, Rosenberg A, Rudikoff S, Singer DS. Expression of a class I MHC transgene: regulation by a tissue-specific negative regulatory DNA sequence element. *New Biologist* 1991;2:1024-33.

Rudikoff S. Principles of tumor immunity: biology of antibody mediated responses. In *Biologic therapy of cancer: principles and practice*, in press.

Shin S-U, DePinho R, Zack D, Rudikoff S, Scharff MD. The instability of immunoglobulin genes in the S107 cell line. *Mol Cell Biol* 1990, in press.

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*, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05596-22 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Pathogenesis of plasma cell neoplasia; resistance and susceptibility genes

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

P.I.:	M. Potter	Chief, Lab. of Genetics	LGN, NCI
	E.B. Mushinski	Bio. Lab. Technician	LGN, NCI
	A. McDonald	Biologist	LGN, NCI
	K. Huppi	Expert	LGN, NCI
	B. Mock	Staff Fellow	LGN, NCI
	E. Shacter	Expert	LGN, NCI
	V. Bohr	Sr. Investigator	LMP, NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

9.0

PROFESSIONAL:

5.0

OTHER:

4.0

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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 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 translocations [rcpt(12;15), rcpt(6;15)] involving directly or indirectly the c-myc locus on chr 15. The principle problems on which we are working are: 1) to identify the genes that determine susceptibility to plasmacytoma induction; 2) to identify additional critical oncogenic mutations that cooperate with c-myc using rapid plasmacytoma induction by infecting pristane-treated mice with transforming retroviruses that contain c-myc and a second cooperating oncogene; and 3) to determine the biological effects of pristane and how it acts in plasmacytoma induction. We have localized genes that determine resistance to plasmacytoma induction finding an exact location of one of the Petr-1 on chr 4. We have previously shown v-abl, v-Ha-ras, and v-raf-1 can cooperate with myc in transforming plasmacytomas and we are now testing new viruses prepared by K. Marcu that contain p53 Ha-ras Ela + H-ras and IL-6 + Ha-ras. Plasmacytomas have been induced with all three. We have developed BALB/c.DBA/2-congenic strains (fv-1^{n/n} N19; Tol Fam3 N12, M.1A N11) that carry various segments of chromosome 4 from the resistant DBA/2 strain. These congenic strains develop fewer plasmacytic proliferative lesions in the first 150 days and a much reduced incidence of plasmacytomas. Enumeration of the foci at between days 120-150 can predict plasmacytoma susceptibility. We are searching for the phenotypic effects of these genes and have evidence that genes involved in DNA repair on chr 4 may be a relevant phenotype.

I. Pathogenesis of plasma cell neoplasia: resistance and susceptibility genes - Dr. Michael Potter

Experiments are designed to elucidate the genetic and biochemical events that occur in the pathogenesis of plasmacytoma development in mice. In this model system plasma cell tumors develop in the mesenteric oil granuloma and are preceded by pre-neoplastic plasma cell proliferations (foci). Factors that influence various stages in plasma cell tumor development can be experimentally determined.

The hypersusceptibility of the BALB/cAn mouse to pristane induced plasmacytomagenesis provides the basis for identifying genes that determine susceptibility. In contrast, the DBA/2 mouse is resistant to pristane induced plasmacytomagenesis. In classical genetic crosses it has been determined that at least 3 different genes determine resistance and susceptibility between these two strains. We have constructed BALB/cAn.DBA/2 congenic strains of mice by introgressively backcrossing various available allelomorph marker genes that distinguish these two strains. After testing a number of these strains we have identified several congenic strains carrying segments of DBA/2 chromosome 4 that have partial resistance to plasmacytoma induction. The major effort of the ongoing studies is to map the locations of these genes on chr-4 and to identify the relevant phenotypes they determine. Focal plasma cell proliferative lesions develop in the pristane induced oil granuloma tissue during the pre-plasmacytoma period (50-150 days). The number of these lesions per mouse is an indicator of susceptibility or resistance. Resistant DBA/2 and CDF1 mice develop few if any of these lesions by day 150 post pristane; further, the C.D2 Tol Fam 3 (chr-4) mice develop a significantly lower incidence of these lesions thereby providing a measurable difference that can be used to determine susceptibility or resistance. Recent results suggest the morphology of the inflammatory response to pristane differs in the chromosome 4 congenics.

We have begun a search for other relevant phenotypic differences that distinguish BALB/cAn from C.D2 congenics. In collaborative studies with V. Bohr, E. Beecham and E. Shacter, it has been found that the C.D2 Tol Fam3 and C.D2 fv-1^{n/n} N19 mice carry gene(s) of DBA/2 origin that determine efficient repair of UV induced damage to the 5' sequences of the c-myc proto-oncogene.

Plasmacytoma induction is influenced by antigenic stimulation. Specific pathogen free BALB/cAnPt mice are refractory to plasmacytoma induction by pristane. This suggests environmental antigens in some way influence the precursor population. Experiments are in progress to test the role of immunization on plasmacytoma induction. Plasmacytomas can also be induced in pristane conditioned mice that have been infected with transforming retroviruses that carry various single or combinations of oncogenes. These viruses have been prepared by Dr. Ken Marcu at Stony Brook and Dr. Ulf Rapp at NCI, FCRF. Viruses carrying deregulated myc oncogenes are not effective while those with abl, raf or ras induce plasmacytomas that have c-myc activating chromosomal translocations (MACTRs). Viruses that carry myc plus a second oncogene (e.g., abl, raf or ras) induce plasmacytomas in pristane-treated mice that do not have MACTRs. We have recently found three new combination viruses that induce plasmacytomas in pristane conditioned mice p53 + Ha-ras, Ela + Ha-ras

and Il-6 + Ha-ras. Dr. Francis Wiener is karyotyping these tumors. These appear to be the first examples of plasmacytomas where c-myc is not directly involved. The requirement for pristane in all but the ABL-MYC virus suggests that the pristane acts by expanding target cell populations. The cooperative effects of these oncogenes in this in vivo system closely parallel similar cooperative effects seen with these oncogenes in fibroblast transformations.

II. The Genetic Control of Plasmacytomagenesis - Dr. Beverly Mock

1. Pristane-induced PCTs in backcross progeny

The inheritance of susceptibility to pristane-induced plasmacytomagenesis is being examined in 750 backcross progeny generated between BALB/cAnPt females (susceptible) and male F1 hybrids (resistant) between BALB/c and DBA/2. Induction studies have been completed on the first 320 backcross progeny. Roughly 12% of the progeny developed PCTs by Day 450, indicating that pristane-induced plasmacytomagenesis is under multigenic control. Of the first 30 backcross progeny which developed pristane-induced tumors, Ls1, Ifa, Sc1, D4Rp1, D4Lgm1 and Mtv-13 on mouse chr 4 were the closest molecular markers cosegregating with PCT susceptibility. A LOD score of 9.03 for cosegregation with Ls1 was considered significant to prove linkage to the mid-distal region of chr 4. So far an additional 30 progeny have developed tumors; these samples will also be subjected to RFLP analysis in an effort to pinpoint the exact location of Pct-1 (susceptibility to plasmacytomagenesis-1) on chr 4.

In addition, other regions of the mouse genome will be scanned by RFLP analysis for the presence of additional susceptibility genes. Special emphasis will be placed on mouse chromosome 11 since the C.D2-Hba/Es-3 congenic exhibits a reduced susceptibility (20% incidence) to tumor formation.

2. Pristane + viral vectors harboring oncogenes

The inheritance of susceptibility vs. resistant phenotypes to tumor formation following the injection of pristane + RIM (a retroviral vector carrying ras, immunoglobulin and myc sequences) was examined in 324 backcross progeny. Forty six percent of the backcross progeny developed tumors indicating that the S/R phenotype could be attributed to a single gene. Analysis of several congenic strains have suggested linkage to chr 17 near H-2. Analyses of backcross progeny are in progress to confirm/reject this chromosomal location.

In addition, pilot studies utilizing pristane plus a retroviral vector carrying abl and myc sequences (AM) have shown that DBA/2 and C3H mice are resistant (0% incidence at Day 150) and BALB/c mice are susceptible (90% incidence at Day 150) to tumor formation. Experiments are planned to determine if the tumorigenic processes induced by pristane + RIM and pristane + AM are under similar genetic control.

III. Biochemistry of the pristane-induced oil granuloma and its role in plasmacytomagenesis - Dr. Emily Shacter

Over the past year, the work has been directed toward three major projects: (1) study unique features of neutrophil-induced DNA damage and repair to try to identify the mutagenic pathways generated by pristane-priming of mice, (2) determine whether inefficient DNA repair in B lymphocytes from BALB/cAn mice may be responsible for their susceptibility to plasmacytomagenesis, (3) identify inflammation-related factors induced by pristane that promote abnormal growth of plasma cells in the oil granuloma.

Major findings:

(1) (Work carried out by Dr. Siegfried Janz) Previous results from our laboratory indicated that neutrophils induce prolonged DNA damage in cocultured B lymphocytes and that repair of this damage is slower than repair of damage induced by relatively simple oxidants such as H_2O_2 . Because neutrophils secrete numerous potentially deleterious compounds in addition to H_2O_2 during the oxidative burst, it is likely that they induce more complex forms of damage which take longer to repair. This could allow time for potentially mutagenic secondary changes to develop in the chromatin. To date, no information is available on the breadth of repair pathways induced by cocubating neutrophils and target cells. To determine whether neutrophil-induced damage initiates activity of excision repair pathways, we have been assaying induction of Unscheduled DNA Synthesis (UDS) in B cells cocubated with activated neutrophils. In the course of establishing suitable assay conditions, it was found that although UDS induced by commonly used reagents such as UV radiation can be measured after an overnight incubation, much shorter repair times are required for the detection of oxidant-induced repair synthesis. The results obtained thus far indicate that neutrophils are potent inducers of UDS in B lymphocytes when the two cell types are cocubated at a 1:1 or 2:1 ratio. UDS was not induced in the neutrophils themselves. This result is interesting in light of the fact that activated neutrophils do repair single strand breaks induced during their own oxidative burst. Further experiments are being carried out to elucidate which elements of the oxidative burst are required for induction of UDS and to compare the results to those obtained with H_2O_2 and with known UDS-inducing agents.

(2) (Work carried out by Jeff Beecham in collaboration with Dr. V. Bohr, Laboratory of Molecular Pharmacology, NCI) A major goal of our research has been to determine whether susceptibility to plasmacytomagenesis may derive from a defect in repair of DNA damage in B lymphocytes from BALB/c mice. Intragenomic repair studies have revealed that in contrast to plasmacytoma-resistant DBA/2 mice, B cells from BALB/c mice are completely deficient in their ability to remove thymidine dimers from the 5'-flanking region of the c-myc oncogene. The observation that B cells from BALB/c mice can remove thymidine dimers in the DHFR gene with equal efficacy to cells from DBA/2 indicates that they are not devoid of the enzymes required to catalyze the repair. In addition, the defect appears to be specific for B lymphocytes insofar as fibroblasts from BALB/c mice are proficient in c-myc repair. Further experiments employing C.D2 congenic strains of mice have shown a

linkage between this in vitro phenotype and partial resistance genes to plasmacytomagenesis located on DBA chromosome 4.

In future experiments, we will apply the UDS assay as an overall excision repair assay in order to gain a broader measure of the extent of the defect in BALB/c. In addition, we will use this assay to try to identify strain differences with damaging agents that may be more biologically relevant than UV to the plasmacytoma system (e.g., neutrophil-induced damage). Finally, we would like to find a faster and less labor-intensive assay that may be used to screen mouse strains for repair efficiency; the intragenomic repair technique that we have employed so far takes two weeks to complete and is not well suited to analyzing large numbers of samples. To these ends, we will begin to set up a new technique that has recently been described that involves using the polymerase chain reaction (PCR) to study gene-specific repair.

(3) a. Most plasmacytoma cells are dependant upon the presence of interleukin-6 (IL-6) in order to grow in culture. This observation has led to the hypothesis that pristane-elicited macrophages, which are an abundant cell type in the oil granuloma, may actively promote the growth of abnormal plasma cells by secreting excess levels of IL-6. In addition, differential levels of growth factor production in susceptible and resistant strains of mice might contribute to the differences in plasmacytoma incidence in the mice. Extensive experiments have been carried out to test this possibility. By employing the B9 cell bioassay, it was found that the concentration of IL-6 in the peritoneal fluid is markedly elevated in pristane-primed BALB/c mice, reaching levels in excess of 1000 pg/ml; in contrast, IL-6 is undetectable (< 10 pg/ml) in the peritoneal fluid of untreated mice. The increase is progressive over time; IL-6 is not detected in the first week after pristane treatment but is found within 4 weeks and increases thereafter. The lag in detection of IL-6 is not caused by the presence of an inhibitor. Serum levels of IL-6 are also elevated in pristane-primed mice but the concentrations are much lower than in the peritoneal cavity. Experiments have been carried out to identify the cellular source of IL-6. Peritoneal exudate cells are fractionated by density gradient centrifugation and by adherence to plastic tissue culture dishes. It was found that cells in the pristane-elicited macrophage fraction, which also contains lymphocytes, secrete low levels of IL-6 constitutively when incubated for several days in a basal serum- and protein-free medium. In contrast, resident peritoneal cells are dormant. In both cases, IL-6 production is dramatically stimulated by various immune reagents such as lipopolysaccharide. Production of IL-6 in plasmacytoma-resistant DBA/2 mice appears to be much lower. The mouse plasmacytoma system may provide a model for studying the regulation of IL-6 production in vivo and for elucidating the role of IL-6 in B cell neoplasia, especially human multiple myeloma and AIDS lymphoma.

(3) b. Pristane-induced plasmacytomagenesis can be completely inhibited by treating the mice with indomethacin in the drinking water. This non-steroidal anti-inflammatory drug can inhibit a number of biochemical pathways but pharmacokinetic studies carried out in our laboratory suggest that the most likely target is the inhibition of prostaglandin synthesis. We are

examining the possibility that pristane-elicited macrophages produce prostaglandins and/or other arachidonic acid metabolites constitutively and that chronic abnormal levels of these compounds lead to immunosuppression and consequent outgrowth of plasma cell tumors. Studies are currently underway to quantify and characterize prostaglandin production from macrophages isolated from control and pristane-treated BALB/c, DBA/2, and CDF1 mice.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08727-14 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Organization and control of genetic material in plasmacytomas

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

P.I.:	J.F. Mushinski	Senior Investigator	LGN, NCI
	G.L.C. Shen-Ong	Senior Staff Fellow	LGN, NCI
	K. Huppi	Expert	LGN, NCI
	H. Mischak	Visiting Fellow	LGN, NCI
	E. Weissinger	Visiting Fellow	LGN, NCI

COOPERATING UNITS (if any)

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Molecular Genetics

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OTHER:

5 1/2

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 (a2) Interviews

B

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

Our research goal is to identify and understand the genes whose altered structure or expression play critical roles in malignancy, immune diseases and normal differentiation. We are concentrating on the expression of a group of these "oncogenes": abl, bcl-2, bcl-3, myb, and myc, as well as the potential oncogenes, Pvt-1 and Protein Kinase C (PKC), 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, and we have shown that this locus is transcribed at very low levels in normal cells but in much higher amounts in some plasmacytomas. Normally Pvt-1 transcripts are ca. 14 kb in length, suggesting that the Pvt-1 locus is very large. In some plasmacytomas with chromosomal translocations in the Pvt-1 locus, the Pvt-1 transcripts are truncated and form chimeras with Ig kappa chain transcripts.

We have isolated a cDNA for mouse bcl-3 (on human chromosome 19) and have mapped it to mouse chromosome 7. During B-cell differentiation its expression is maximal in cells that have not undergone Ig isotype switch. This suggests that the chromosome translocations involving this locus that characterize some CLL's occur in this stage.

The ABL-MYC retrovirus, expressing v-abl and c-myc, rapidly induces intraperitoneal plasmacytomas in BALB/c and other strains of mice, even in the absence of i.p. oil and in the absence of helper virus. If the mice are preimmunized with lysozyme or sheep red blood cells, about 60% develop tumors producing antibody specifically directed against the immunogen. This may prove to be a useful alternative to hybridoma technology for generating monoclonal antibodies.

Major Findings:

I. Gene Expression

A. Expression of cbl, Pvt-1 and bcl-3 oncogenes and PKC family of genes in normal and neoplastic hemopoietic cells. cbl, Pvt-1 and bcl-3 genes have been implicated in oncogenesis of B lymphocytes: cbl in a mouse lymphomagenic virus, Pvt-1 in chromosome translocations characteristic of mouse plasmacytomas and human Burkitt lymphomas, and bcl-3 in t(14,19) translocations found in human CLL. The expression of these genes differs strikingly, however, in experimental mouse tumors. The steady-state levels of cbl mRNAs are practically invariable among hemopoietic tumors and in mitogenically stimulated lymphocytes and fibroblasts. In sharp contrast, the RNA levels of bcl-3 and Pvt-1 rise transiently following mitogenic stimulation and vary significantly in lymphomas representing different stages of B-cell differentiation. The different patterns of expression are consistent with the hypothesis that non-random, transforming chromosome translocations occur at sites on the two chromosomes at times when the genes are simultaneously being expressed, i.e., at the same stage in a cell's maturation. 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 coamplified 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. Some aspects of the expression pattern of bcl-3 resemble that of bcl-2, a proto-oncogene that is activated by chromosomal translocation in a different group of human B lymphomas, so it will be important to determine how similar or different these two genes are in function.

Expression of Protein Kinase C (PKC) in mouse hemopoietic cells is a complex issue, inasmuch as there are at least 7 different genes encoding 7 or more different, but similar, serine/threonine protein kinases. To perform these experiments we have cloned complete cDNAs of mouse PKC- δ , - ϵ , - η and - ζ , and used PCR to generate isoform-specific partial cDNA probes for the remaining isoforms. Our studies indicate that PKC- δ is the most abundantly expressed PKC isoform in hemopoietic cells, while PKC- ϵ and - ζ are expressed in many cells, but only at low levels. The other isoforms are expressed variably, depending on the type of cell. Mouse myeloid tumors, unlike certain human myeloid cells, express virtually no PKC- α or - β , and, instead, express almost exclusively PKC- δ . PKC- η appears to be the principal isoform expressed in T cells. PKC- α and - β are expressed in B lymphocytes in a stage-specific manner. That is, the most terminally differentiated of the B cells, exemplified in plasmacytomas, have the lowest levels of PKC- β RNA and a prominent band of PJC- α with an unusually short size, 2.5 kb. Other expression data suggest that B cell differentiation may be affected by the change in expression of PKC- α and - β , and this hypothesis is being tested. Analysis of PKC expression at the protein level with isoform-specific antibodies confirmed the expression data obtained with RNA levels, indicating that expression is regulated at the transcriptional level.

B. Complimentation of over-expressed c-myc by v-abl in ABL-MYC retrovirus. Abelson murine leukemia virus (A-MuLV) induces tumors in a limited number of inbred mouse strains, and these tumors are usually made up of pre-B lympho-

cytes. The myc oncogene, when overexpressed in cells as a retrovirus, principally induces tumors of the myeloid lineage. When both oncogenes are coexpressed in mouse cells infected in vivo or in vitro with the ABL-MYC retrovirus, exclusively plasmacytomas are obtained in every animal within only a few weeks. In order to make use of these properties, we immunized BALB/c mice with either hen egg lysozyme (HEL) or sheep red blood cells prior to inducing tumors with ABL-MYC. 100% of the mice developed plasmacytomas of which 60% secrete antibodies specifically directed against the immunogen. These tumors may be polyclonal or monoclonal, and their transformed cells can be detected as early as 12 days after virus injection. 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. The tumor cells can be transplanted into syngeneic mice, or grown in vitro, initially using a mouse fibroblast feeder layer plus IL-6. Oligoclonal tumors can be separated into monoclonal cultures by limiting dilution, if desired. It is hoped that experiments currently in progress will show that this combination of oncogenes can be used to immortalize antibody-secreting cells in other strains of mice and in other species.

C. Transformation and transcription activation by the myb proto-oncogene. Forms of myb that have been truncated by retroviral insertion have been shown by us to be involved in myeloid tumor development in BALB/c mice given i.p. injections of oil. We have demonstrated that both mice and men have alternate forms of myb proteins secondary to alternative RNA splicing. We are now testing whether these alternative forms of myb transactivate target genes similar to or different from the major form of c-myb.

II. Studies of hereditary chronic multifocal osteomyelitis (CMO).

A mutant line of inbred mice develop chronic granulomatous inflammation of the small tubular bones of the tail and limbs. This disease has been shown to be determined by a single autosomal recessive gene of mouse chromosome 18. Using gene cloning in bacteriophages and yeast artificial chromosomes, attempts are underway to identify this gene and characterize its products and its physiology. It is anticipated that this gene may play an important role in the development of or proliferation of myeloid cells. It is hoped that further insights into its structure and function will contribute to our understanding of chronic inflammatory processes.

III. Immunoglobulin isotype switching to make possible IgD secretion.

In order to learn how IgD secretion is accomplished, we isolated and studied 2 BALB/c plasmacytomas as well as 9 BALB/c and 3 C57BL hybridomas that secrete IgD. Studies of DNA from these cells indicate that the IgM gene is deleted from all the lines, even though it is known that mouse IgD lacks typical repetitive "switch" sequences in its 5' flank. Sequence studies of the switch recombination junction in these 14 cell lines showed that these sequences were similar to those of other heavy chain genes. This suggests that non-homologous, illegitimate recombination is utilized in switch to IgD as well as to other heavy chain isotypes which have repetitive 5' "switch" sequences.

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

PROJECT NUMBER

Z01 CB 08950-09 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular and biological basis of immune recognition

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

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PROFESSIONAL:

3.5

OTHER:

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

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

Protein-protein interactions underlying molecular recognition are studied, utilizing antibodies (mAbs) specific for the protein hen egg white lysozyme (HEL), a protein which has long served as a prototype for investigating the specificity of immune recognition. In order to understand the molecular basis of interaction between an antibody and a protein antigen, the details of three structures must be defined: the antigenic epitope, the complementary paratope, and the interface of the antibody-protein complex. Mapping the epitopes recognized by a series of mAbs specific for HEL has allowed partial definition of the antigenic structure of this protein. Our results indicate that antigenic structure of HEL, as recognized by the immune system, is an emergent property which changes during the course of the immune response. The X-ray structure of the HyHEL-26 Fab-HEL complex has been solved and is being refined, bringing total structures of mAbs from our laboratory to 3. The HyHEL-26 epitope is nearly identical to that recognized by HyHEL-10, and the primary and genetic structures of HyHEL-26 are closely related to those of HyHEL-8 and -10. These results represent the first time structures have been obtained for 2 structurally related antibodies complexed to the same protein epitope, and we now have an unprecedented system for studying structure-function relationships. We have begun to experimentally investigate structure-function relationships in the interface by site-specific mutagenesis of both antigen and antibodies. These studies are beginning to define fundamental principles that will allow prediction of function from structure, principles that are critical to such applications as antibody design and vaccine development. We are also approaching the problem of vaccine development by investigating immunogenicity and protective epitopes in *Shigella flexii*. In addition, we have begun investigations which suggest a significant influence of antigenic exposure on development of the specificity repertoire and plasmacytomagenesis.

Major Findings

A. Molecular Basis of Immunological Recognition in Antibody-Protein Interactions

1. The molecular basis of antibody complementarity -- Dr. Smith-Gill, Mr. Drew Newman (graduate student), in collaboration with Dr. Konrad Huppi

An X-ray structure of the HyHEL-26 Fab-HEL complex has been solved by the laboratory of Dr. Steven Sheriff (Bristol-Meyers-Squibb) and is being refined, bringing the total number of Fab-HEL structures of antibodies from our laboratory to 3. Specificity mapping with HyHEL-26 indicated it had an epitope very similar to, if not identical with, that recognized by HyHEL-10, although it is of slightly lower affinity and much more sensitive to mutations within the epitope than is HyHEL-10 (which in turn is of slightly lower affinity and more sensitive to mutations in the epitope than is the structurally and functionally related mAb HyHEL-8). A low resolution (~6 Å) structure of the HyHEL-26 Fab-HEL complex, obtained last year in the laboratory of Dr. Sheriff, indicated that HyHEL-26 recognized a region very close to that recognized by HyHEL-10. Efforts since that time have focused on production and purification of additional HyHEL-26 in order to obtain a higher resolution structure, and Dr. Sheriff's laboratory has recently obtained a moderate resolution (~4 Å) X-ray structure of Fab HyHEL-26 complexed with HEL, which confirms that the epitope is nearly identical to that recognized by HyHEL-10. In addition, the sequences of PCR clones of the H and L chain V regions indicate that HyHEL-26 is genetically and structurally closely related to HyHEL-10 and HyHEL-8. Since the HyHEL-26 hybridoma was produced nearly 10 years later than HyHEL-8 and HyHEL-10, these results provide the first strong support for the hypothesis that there are a restricted number of structural and underlying genetic solutions that provide functional complementarity for a given protein epitope, an extension of similar observations which have been made by a number of laboratories for a variety of haptenic antigens. These results also represent the first time structures have been obtained for 2 structurally related antibodies complexed to the same protein epitope. We now have an unprecedented system for interpreting the structural correlates underlying affinity and specificity, because we are in a unique position of being to manipulate *both* antigen and antibody in 2 related complexes with structural definition. Experiments with the HyHEL-26 complex will be an extension of site-directed mutagenesis experiments already in progress to dissect the HyHEL-10-HEL functional interface. HyHEL-10 has previously been cloned, expressed, and subjected to site-directed mutagenesis in my laboratory by Dr. Tom Lavoie (currently at Bristol-Myers Squibb). Several other mAbs whose preliminary fine specificity profiles indicate they also bind the same epitopic region as HyHEL-8, -10, and -26 are currently being characterized in greater detail.

We are also investigating in detail a panel of newly characterized mAbs which are functionally related to HyHEL-5. Drew Newman has identified 5 newly characterized anti-HEL mAbs (HyHEL-501 thru 505) which recognize HEL residue Arg68, with specificity profiles that are nearly identical to that of the structurally defined HyHEL-5, but whose affinities range over 2 orders of magnitude. In addition, he has identified 2 mAbs (HyHEL-47 and -48) that clearly recognize HEL residue Arg68, and whose functional epitopes are clearly overlap with but are distinct from those of HyHEL-5 and the HyHEL-500 series. He is currently producing the PCR clones of the H and L chain V regions of these 7 mAbs, in collaboration with Dr. Konrad Huppi, in order to obtain their primary nucleotide and amino acid sequences. These mAbs will be characterized both structurally and functionally, in order to investigate the range of possible structural and functional solutions for specifically binding a defined antigenic region. We hypothesize that these mAbs represent at least 2 different structural groups, corresponding to the 2 distinct functional groups (HyHEL-5 and 500's, vs. HyHEL-47 and -48).

2. Antigenic structure and the molecular basis of antigenicity -- Dr. Smith-Gill, Mr. Newman, Dr. Gabrielle Tuscherer

Mr. Newman has utilized patterns of antibody overlap to probe the antigenic structure of HEL, which appears to be an emergent property that changes during the course of the immune response. Results with 50 BALB/c IgG anti-HEL mAbs representing different stages during the course of the immune response indicate

that average avidity does not increase during the immune response, but range of avidity and diversity of specificity do increase. Thus, affinity maturation of IgG serum antibodies appears to represent an increase in antibody concentration and diversity (which would allow for possible synergistic interactions to form antibody complexes) rather than increasing average avidity of individual antibody clones. Late antibodies (late secondary and hyperimmune) recognize epitopes that are grouped into larger, nonoverlapping antigenic regions with definable functional boundaries. The apparent antigenic regions show a rough correspondence with the tertiary structure of HEL. In contrast, the majority of early antibodies (primary and early secondary) fall into 3 groups: (i) a set localizing to Complementation Group III of late response antibodies (groups I and II are not represented in early response antibodies); (ii) a group which recognize at least one region which is distinct from and nonoverlapping with the antigenic regions recognized by late antibodies, and (iii) a smaller group which all overlap significantly with 2 or more of the late response antigenic regions. A similar antigenic structure is defined by early response mAbs from BALB.k H-2 congenics, indicating that at least the early response pattern does not depend upon H-2 haplotype. The results also suggest clonal replacement during the course of the immune response, although affinity does not appear to be the selective factor.

While the total panel of mAbs cover a large portion of the HEL accessible surface, consistent with the hypothesis that most if not all of the accessible surface is antigenic, the apparent existence of functionally nonoverlapping regions was not expected. Furthermore, at least one pair of functionally nonoverlapping mAbs, HyHEL-10 and D1.3, have X-ray defined "structural" epitopes which do overlap significantly, raising the question of the relationship of the functional epitope to the structural epitope. Several testable hypotheses may explain these observations: (i) antigenic structure reflects an inherent property dependent upon the structure of the protein; (ii) the emergent antigenic structure reflects immune regulation, particularly antigen processing and presentation; (iii) the emergent antigenic structure reflects determinant selection by B-cells, such as epitope-directed antigen-processing by B cells. Dr. Gabrielle Tuscherer is beginning experiments to test these hypotheses, in collaboration with Dr. Ann McDonald.

Our results to date indicate that high affinity mAbs specific for a globular protein like HEL will have little, if any, meaningful cross-reactivity with linear peptides. However, it should be possible to geometrically constrain a peptide sequence in order to match the conformation of the cognate sequence on the native protein. If a conformationally constrained peptide can be made that will bind a structurally defined antibody it should give insight into the minimal structural requirements for functional complementarity. Experiments are in progress to design such peptides, in collaboration with Dr. Gary Glick (University of Michigan) and Dr. Manfred Mutter (Lausanne University). Dr. Tuscherer has previously synthesized conformationally constrained Template Assisted Synthetic Peptides (TASP), modeled after the α -helix which is the HyHEL-10 epitope. A problem encountered in assessing antibody binding to synthetic peptides is assay sensitivity, which may not be sufficient to detect low affinity binding, and the lack of suitable controls for positive binding. Future experiments will be focused on developing more sensitive binding assays.

3. Analysis of the Antibody-Antigen Interface -- Dr. Smith-Gill

We have continued detailed analysis of the HyHEL-10 Fab-HEL interface by site-directed mutagenesis of both the antibody and the antigen. Dr. Lauren Kam-Morgan, in the laboratory of Dr. Jack Kirsch (U Cal, Berkeley), has produced and expressed in yeast a series of site-directed mutants of HEL at residues that are within the HyHEL-10 epitope, as defined by the HyHEL-10 Fab:HEL X-ray structure. Eleven mutant lysozymes have been tested for reactivity with HyHEL-10 in a competitive inhibition assay, and by utilizing catalytic activity of HEL to estimate uncomplexed (unbound) enzyme in sequential saturation assays to estimate K_D . The latter estimates are utilized to calculate the free energy (ΔG_{assoc}) and the change in free energy ($\Delta\Delta G_{\text{assoc}}$) caused by specific mutations. The results to date indicate: (i) the effects of site-directed mutations of the antigen appear to be local, in contrast to site-directed mutations of the antibody which we have shown previously can produce indirect or long-range effects; (ii) the effect of mutations on binding can vary qualitatively with the substitution and the antibody; (iii) a given substitution may produce both direct and indirect (steric) effects on binding; (iv) experimental estimates of ΔG contribution by a given residue give

good qualitative agreement with theoretical calculations of ΔG made by Dr. J. Novotny (Bristol-Myers Squibb Pharmaceutical Research Institute) from the X-ray coordinates. These experiments, which are continuing, allow correlation of the functional role of each residue with its apparent structural role, and provide the beginning of a theoretical basis for prediction of functional complementarity from structural data.

B. Characterization of Protective Immunity to *Shigella flexii*: A Model System for Vaccine Development – Dr. Smith-Gill, Mr. Mainhart, in collaboration with Dr. Antoinette Hartman (Department of Biologics Research, Walter Reed Army Institute of Research)

In order to begin application of the principles deriving from our studies on the HEL model system, we have initiated a study, in collaboration with Dr. Hartman, to investigate the nature of the protective immune response to *Shigella flexii*. Dr. Hartman has previously developed a guinea pig model for vaccine testing and studying the immune response to this pathogen. If bacteria are allowed to invade the eye mucosal tissue of guinea pigs (Sereny test), they develop a strong protective immunity to subsequent challenge. We have utilized a similar immunization protocol with BALB/c mice, and made hybridomas using splenocytes from immunized mice to form hybridomas. The resulting hybridomas included IgG, IgM, and IgA secreting clones. One hybridoma produces IgG mAb which strongly and specifically reacts with *Shigella* LPS. We are currently developing PCFIA assays for rapid screen in the Screen Machine, and characterizing other clones. The long-term goals of this project, in which Mr. Charles Mainhart is taking a leadership role, include: (i) to test mAbs specific for LPS and other *Shigella* antigens for ability to confer protective immunity when administered passively to guinea pigs; (ii) to identify the determinants recognized by any protective mAbs; (iii) to "humanize" any mAbs which confer strong protective immunity in an animal model for possible clinical trials.

C. The Influence of Antigenic Exposure on Immunopathological Responses

1. Influence on Plasmacytomagenesis and T Cell Function -- Dr. Ann McDonald, in collaboration with Ms. Linda Byrd (graduate student)

Dr. Ann McDonald, in collaboration with Ms. Linda Byrd, is continuing a systematic study of the role of T cells in pristane-induced plasmacytomas. She has previously shown that plasmacytoma-susceptible BALB/cAnPt (BALB/c) mice develop an infiltration of Thy-1⁺, CD4⁺ lymphocytes in pristane-induced peritoneal exudate and oil granulomas 50 days or more after the intraperitoneal injection of pristane. Plasmacytoma-resistant DBA/2N and (BALB/c x DBA/2N)F1 mice develop oil granulomas but no T helper cell infiltration (manuscript submitted for publication). Recently, she demonstrated that the increase in CD4⁺ cells was neither significantly affected by a second injection of pristane in BALB/c mice nor was induced by a second injection in DBA/2N mice. Moreover, indomethacin, which has been shown to prevent the development of plasmacytomas in BALB/c mice prevented the infiltration of CD4⁺ cells. Future work will focus on: (i) isolating the T cells and identifying cytokine secretion patterns to ascertain how the T cells may be promoting plasmacytome development, and (ii) in vivo depletion of CD4⁺ cells to determine whether T cells are an absolute requirement for plasmacytomagenesis.

Ms. Byrd had previously found that when BALB/c mice were converted to Specific Pathogen Free (SPF) status, the incidence of pristane-induced plasmacytomas dropped from 50% to less than 5%. FACS analysis by Dr. McDonald of SPF-BALB/c oil granulomatous tissues revealed a significantly smaller influx of CD4⁺ cells than in conventionally (CON) housed mice. Moreover, while both CON- and SPF-BALB/c mice had similar patterns of gut flora colonization, only CON-BALB/c mice had occasional circulating antibodies to Mouse Hepatitis and Sendai viruses. The results suggest that plasmacytoma formation depends upon exogenous, possibly viral, antigenic stimulation and that the presence of minimal gut flora is insufficient to render these mice susceptible to plasmacytoma induction. SPF-BALB/c mice are widely used in immunological research. The above findings suggest that while these mice may be capable of responding to a variety of antigens, the immune response may be altered as a result of prolonged antigenic deprivation (manuscript submitted for publication).

Recent studies are focusing on the ability of the SPF immune response to respond to a variety of antigenic stimuli. For example, we have already found that lymph node cells from SPF-BALB/c mice injected subcutaneously with hen egg lysozyme in complete Freund's adjuvant do not proliferate as well as CON-BALB/c mice when cultured *in vitro* with antigen. Mitogenic responses to Con-A are unaffected. Cytokine production and cytotoxic T-cell response are also being monitored (in collaboration with Dr. V. DeLaCuz, Medimmune, Inc.), and the influence of specific routes of antigenic delivery on T-cell function.

2. Influence on B-cell Repertoire – Dr. Smith-Gill, Mr. Mainhart, Dr. McDonald, in collaboration with Ms. Byrd

Comparison of hybridomas derived from spleens of HEL-immunized SPF and CON BALB/c mice indicates that the B cell response is underdeveloped in SPF compared to CON mice: (i) Total IgG serum levels in SPF mice are less than 10% that of CON mice in our colonies, even after repeated immunizations with HEL or ovalbumin in Freund's adjuvant; (ii) Although IgG and IgM secreting hybridomas were isolated in approximately the same proportion from mid and late secondary response SPF and CON mice, less than 1/3 as many secreting clones were isolated from the SPF mice; (iii) Approximately half of the SPF IgM mAbs were HEL-reactive, and 2/3 of these were HEL-specific, in contrast to CON mice where not a single IgM mAb was specific for HEL at any point in the immune response, and even polyreactive IgM mAbs which bound HEL were rare; (iv) a much higher percentage of the IgG mAbs from SPF mice were HEL-specific, and, unlike previous results with CON mice, no polyreactive IgG's were found in the SPF mAbs. Overall, these results suggest a smaller pool of B-cells to be either specifically antigen-activated or to be activated as "bystanders".

Experiments are continuing to define the role of antigenic stimulation on maturation of the B and T cell compartments, and to identify the specific antigen-driven steps which are critical to development of PCT susceptibility.

D. Plasmacytoma Induction by *v-abl* – *c-myc* Retrovirus – Dr. Evi Weissinger, in collaboration with Dr. Mushinski

Dr. Evi Weissinger is continuing investigation of PCT induction by the ABL-MYC virus. Pilot experiments had indicated a significant difference in latency of ABL-MYC induced PCT-development between unprimed BALB/c and BALB/c *nu/nu* mice; PCT in the nude mice developed about 30 days earlier. These results, which are currently being repeated, suggest that T cells might have an influence on latency of tumor development.

Dr. Weissinger had previously demonstrated that if mice were immunized with HEL prior to infection with ABL-MYC virus, a high proportion (~ca 60%) of the induced PCTs were HEL-specific, suggesting that the target of this virus was an antigen-committed, mature B cell. She has successfully infected lymphoid cells directly with the virus *in vitro*, and has demonstrated that target cells can be found in every lymphoid organ and in the peripheral blood; recipients transplanted with different lymphoid organs infected *in vitro* developed PCT in most (~90%) cases, but with varying latencies. To date, antigen-priming of animals prior to infection with virus led to the development of only one antigen-specific PCT; experiments are in progress to improve the frequency of antigen-specific PCT with different target organs, a tool which would prove very valuable in the study of B-cell functional compartments. She is collaborating with Dr. Mushinski to explore utilization of this virus as a useful tool for generating antigen-specific mAbs with unusual biological properties or from lymphoid compartments or non-murine species, that are difficult to target to utilizing conventional hybridoma technology.

Publications

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

PROJECT NUMBER

Z01 CB-08952-05 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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, laboratory, and institute affiliation)

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

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TOTAL MAN-YEARS:

3.8

PROFESSIONAL

3.8

OTHER:

0.0

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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 overall objective of the laboratory is to develop mouse model systems for studying acute myeloid leukemia. With the use of different retroviruses several leukemias within the monocyte-macrophage lineage have been induced in mice undergoing a chronic inflammatory response. Since the effect of oncogene activation in these leukemias is a major interest of the laboratory, two types of studies are being pursued in this area at the present time. One study is aimed at examining the various mechanisms by which c-myb can be activated by viral insertional mutagenesis. Insertional mutagenesis by Moloney murine leukemia virus (MuLV) in our promonocytic leukemias results in very characteristic RNA and protein products, however insertional mutagenesis of c-myb by FB29 is only partially characterized. So far we have demonstrated that the c-myb protein products from these leukemias are unique. They probably result from unique hybrid RNAs in which gag and myb sequences are juxtaposed and these are presently being characterized in more detail. There is also an ongoing interest in insertional mutagenesis at other sites in the genome. Clonal integration sites of Moloney MuLV, Ampho 4070, and FB29 into the mouse genome of leukemias have been characterized and selected integration sites have been cloned into lambda phage. Cloned sequences will be used to search for common integration sites amongst leukemias. Another focus of the laboratory is the delineation of specific stages of disease development and trafficking of preleukemic cells. A RNA-PCR technique for detection of a molecular marker in leukemic cells has provided some information about trafficking patterns. Leukemic cells can be detected in hematopoietic organs such as bone marrow, liver, spleen and mesenteric granuloma as early as 3 wks post viral infection even though the disease is not evident until 3-4 mo. Although pristane increases the number of mice in which such cells can be detected, they can be detected even in the tissues of some mice that have not been treated with pristane. Future experiments will be aimed at extending our knowledge about trafficking and the role of hematopoietic organs in this disease.

Major findings:

Activation of the c-myb gene by insertional mutagenesis: molecular comparison of activation by different viruses. Three retroviruses, Moloney MuLV, Ampho 4070 and FB29 have been shown now to induce promonocytic leukemias through insertional mutagenesis of the protooncogene, c-myb. Studies on the mechanism of activation, for those induced by Moloney MuLV, have shown that, in association c-myb activation, are altered c-myb transcription and truncation of the c-myb protein at its amino terminus. Hybrid gag-myb RNA is formed from an aberrant splicing event that depends upon the utilization of a cryptic donor splice site in the retrovirus gag gene. C-myb protein translation initiates at a methionine in gag which is not utilized by the virus during its replication. At present we do not know the exact mechanisms by which Ampho 4070 and FB29, a strain of Friend MuLV activate c-myb. A recent analysis of the FB29 induced tumors, with c-myb rearrangements, however, indicate that the c-myb proteins expressed in these tumors have unique sizes when compared to the normal c-myb protein or leukemia-specific proteins observed previously. It is hypothesized that they derive from uniquely rearranged retroviral and c-myb genes. DNA fragments overlapping the presumed gag-myb junction are being amplified from these leukemias by the polymerase chain reaction and will be sequenced to determine the mechanism of activation.

Search for new genes activated by insertional mutagenesis in promonocytic neoplasms. A recent examination of viral integration sites in promonocytic leukemias has been performed and two observations have been made. One is that one third of all Amphotropic virus induced tumors in DBA/2 mice, which do not have rearrangements of the c-myb or c-myc loci, can be demonstrated to have one or more clonal retroviral integrations. Second, BALB/c leukemias with c-myb activation by insertional mutagenesis invariably have one or more additional integrations. This has led us to consider the possibility that insertional mutagenesis may be resulting in activation of additional oncogenic sites in the cellular genome. We have cloned in Lambda phage fragments containing viral insertional sites from two amphotropic virus (Ampho 4070)-induced leukemias and are preparing cellular-derived probes. These probes, as well as others to be derived in a similar manner, will be utilized to search for common integration sites amongst leukemias. We are interested in common sites of integration because they could represent protooncogene containing regions of the genome.

Retroviral genes specifically required for promonocytic leukemia induction. Our data has shown that promonocytic leukemia induction in Moloney MuLV-infected mice undergoing a chronic inflammatory response, requires certain sequences in the structural gene region of Moloney MuLV. The required sequences are not found in Friend MuLV, (clone 57) a virus which does not cause promonocytic leukemia. To determine more specifically the required sequences in the structural gene region of Moloney MuLV we are analyzing a number of Moloney/Friend MuLV reciprocal recombinants in each of the major structural genes (gag, pol, env) in collaboration with M. Sitbon, Paris. For each of these viruses, we are determining infectability of the viruses as well as their ability to induce promonocytic leukemia in the presence of a pristane-induced inflammatory response. Results of env and pol reciprocal recombinants so far suggests that the pol gene does not play a role in determining the ability of the virus to cause this disease. However, we are still testing additional recombinants

which should determine whether env or gag sequences or env and gag sequences contain the required elements in Moloney that allow it to be pathogenic for myeloid cells.

Preleukemic phase of Moloney MuLV-induced acute promonocytic leukemias.

Promonocytic tumors resulting from intravenous (i.v.) inoculation of Moloney MuLV into pristane-treated mice are apparent in the peritoneal cavity after a latency of 2-3 mo. The i.v. mode of injection is critical for disease induction and suggests that the cells that ultimately become transformed are infected early on by the virus within organs that are readily accessible by i.v. injection. Our laboratory has been interested in the preleukemic events that might occur in hematopoietic organs such as the bone marrow and spleen prior to the outgrowth of fully transformed cells in the peritoneal oil granuloma. In addition we have been examining the role of pristane in influencing the hematopoietic environment in such organs, since it may facilitate leukemogenesis by indirect mechanisms. Our experiments have shown that, following pristane treatment, the number of progenitor cells in the spleen committed to the granulocytic/macrophage lineage are significantly increased. In addition, it was found that splenectomy, either pre or post virus infection, significantly decreases the incidence of disease. In order to determine more about the role of the spleen and other hematopoietic organs during the early preleukemic phase (2-3 months post-virus infection), we have developed a PCR technique for monitoring the presence of preleukemic cells in which myb activation has occurred (100 percent of the Moloney MuLV promonocytic leukemias examined to date in BALB/c have undergone retrovirus-related mutagenesis of their c-myb genes). The technique involves reverse transcription of cellular mRNA and amplification of the gag-myb junction characteristic of this leukemia using primers from the gag and myb genes on either side of the junction. Amplified products of 289 bp and 328 bp are separated on agarose gels and detected using 18-mer oligonucleotide probes that bridge the viral-myb junctions; two different junctions have been found to exist in these leukemic cells depending upon whether the viruses integrate upstream of UEl or vEl. We first worked the technique out using cultured leukemia cells and have found that the probe is highly specific for transformed cells and that we can detect at least one transformed cell in 10^5 cells. In vivo experiments are now underway in which we are examining tissues at various times after viral infection for the presence of the unique PCR products and we have found that cells with the characteristic marker can be found in hematopoietic organs as early as 3 wks post virus inoculation. In the analysis of mice infected for 3 wks we included 4 mice that had been treated with pristane three weeks prior to injection of virus and 4 mice not treated with pristane. Interestingly, in this 3 wk group, 4 out of 4 mice treated with pristane showed evidence of preleukemic cells in the bone marrow compared to 1 out of 4 mice not treated with pristane and 2 out of 4. In addition 4 out of 4 pristane-treated mice had preleukemic cells in the spleen and liver respectively compared to 0 out of 4 and 1 out of 4 mice not treated with pristane. Therefore, it appears that the virus is able to integrate and activate c-myb in the absence of pristane treatment but that the number of cells present this early with the altered c-myb transcript is increased in mice that had been injected with pristane. This effect could be due to either increased number of integration events involving the c-myb locus or an increased

proliferation of cells already having an activated c-myb locus. Another interesting observation that was made was that preleukemic cells are present in the mesentery of mice not treated with pristane as well as in the granuloma-laden mesentery of the pristane-treated mice. Additional analysis of tissues from mice at various times post viral infection and in different strains of mice will assist in delineation of specific stages of neoplastic disease, in determining the trafficking of leukemic cells during disease and the role of pristane in the different stages. In addition, we will be able to determine if the resistance to disease observed in many strains of mice might be related to an inability of the virus to activate c-myb.

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 in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08953-01 LGN

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Effects of individual genes on hematopoietic cell differentiation and function

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

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TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

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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.)

Research in two areas has continued, namely CD45 isoform expression during B lymphocyte and myeloid differentiation, and the effects of the mutant genes lpr and gld on T cell development and function. By PCR analysis and immunoprecipitation we showed that the 220 kDa isoform of CD45 was expressed on B lineage cells from pro-B cells to B immunoblasts. Differentiation into plasma cells was accompanied by a switch to two lower MW isoforms, a predominant species that spliced out exons 4, 5 and 6 and a minor species that spliced out two of the three exons. By comparison, myeloid cells spliced out exons 4, 5 and 6 early in development and retained this phenotype in mature cells. Future studies will focus on the functions of the various B lineage CD45 isoforms. To further evaluate the effects of lpr and gld on T cells we examined surface antigen expression and cytokine production by four T cell subsets. We demonstrated that a high proportion of CD4⁺ and CD8⁺ T cells expressed high levels of CD44 and reduced levels of Mel 14 and CD45R, a phenotype consistent with T cell activation. These two subsets also hypersecreted IL-2, IFN- γ and TNF following stimulation. By comparison, the two unique B220⁺ T cell subsets had the phenotype of less mature T cells were refractory to stimulation and did not secrete cytokines. Future research on this project is directed toward gaining insight into the mechanisms governing the functional energy of some lpr and gld T cell subsets and the apparent hyperactivity of others; the developmental relationships among the various T cell subsets, and the precise role of CD4⁺ and CD8⁺ T cells in the genesis of lymphoproliferative disease.

Major Findings:

In preceding annual reports two areas of research of longstanding interest were outlined. The first was a study of CD45 expression during the development of B-lymphocytes and macrophages. The second was the continuation of a long term study to determine the mechanisms by which the mutant genes lpr and gld exert their influence on T cell growth and function. Work on both of these projects has continued and the progress made is summarized below.

A. CD45 Expression During B Cell and Macrophage Differentiation

CD45, a cell surface glycoprotein encoded by a single gene, is expressed in abundance on the majority of nucleated haematopoietic cells. Distinct isoforms of CD45 are expressed on various cell lineages. For example, the predominant isoform precipitated from B cells is 220 kDa and from macrophages is 205 kDa. More heterogeneity is observed between T cell subsets with CD4⁺ T cells expressing 190 kDa and 180 kDa isoforms and CD8⁺ T cells expressing 220, 205 and 190 kDa isoforms. Changes in CD45 isoform expression also have been reported during early T cell development in the thymus and following activation of mature peripheral T cells. The different isoforms are derived by alternative splicing of exons 4, 5 and 6 in the external domain and by further post-translational modification. All isoforms share a membrane-spanning domain and a large cytoplasmic tail that recently was shown to have protein-tyrosine phosphatase activity. Although the functions of the various CD45 isoforms are not well understood, recent evidence indicates that CD45 can associate with other molecules (e.g., Thy-1, CD3-zeta and CD2) and may play an important role in T cell signalling. In contrast to T cells, little is known about CD45 expression or function in B lymphocytes and macrophages or their progenitors.

Because CD45 is potentially of universal importance in haematopoietic cell differentiation and function, we were particularly interested in re-examining the expression of this molecule on cells of the B and macrophage lineages. Our early studies of CD45 expression on B lineage tumors representing cells arrested at different stages of development from progenitors to plasma cells, indicated that while all cells bound the pan-reactive mc anti-CD45 Ab, F-11, some pre-B cells and most plasma cells did not react with the mcAb, 6B2 that recognizes the 220 kDa B cell-restricted isoform of CD45. In addition, we reported that the macrophage cell lines derived from the bi-potential progenitor lines, HAFTL-1 and HAFTL-3, had a smaller size (4.7 Kb) CD45 mRNA species than the precursors (5.1 Kb) and were no longer recognized by mcAb 6B2 but were still reactive with mcAb F-11. These data implied that B cells and macrophages also express different CD45 isoforms during development. To evaluate the isoform differences at the molecular level, mRNA was extracted from a panel of B lineage tumors representing progenitors, precursors, mature B cells, immunoblasts and plasma cells and the sizes of the CD45 mRNA species compared by Northern blot analysis. These studies revealed that the predominant mRNA species in B cell progenitors and mature B cells was approximately 5.1 Kb in length whereas plasmacytomas contained a consistently smaller (4.7-4.9 Kb) CD45 mRNA transcript.

To more accurately analyze the splicing patterns occurring during B-lymphocyte development, we utilized the sensitive technique of PCR analysis. PCR products were visualized first by ethidium bromide staining and then were transferred to Nytran. The Southern blots were hybridized sequentially with oligomer probes for exons 3 through 8. The predicted band sizes for all possible combinations of exons are as follows: Exons 4 + 5 + 6 (534 bp); 4 + 5 (393 bp); 4 + 6 (387 bp); 5 + 6 (405 bp); 4 alone (246 bp); 5 alone (264 bp); 6 alone (258 bp); 4, 5 and 6 deleted (117 bp). These studies demonstrated that pre-B cells and mature B cells have a predominant 534 bp band that by hybridization contained exons 4, 5 and 6. In addition, HAFTL-1 and HAFTL-3 had a smaller band of approximately 400 bp that hybridized strongly with the exon 5 and 6 probes and only weakly with the exon 4 probe indicating that it was composed predominantly of exons 3, 5, 6, 7 and 8. By comparison, of nine plasmacytomas analyzed, all lacked the 534 bp band and had a predominant 117 bp band that did not hybridize with exon 4, 5 or 6 probes. Three other bands were in evidence, a fairly abundant broad band of approximately 250 bp that hybridized with exon 4, 5 and 6 probes that probably represents unresolved bands of 246, 264 and 258 bp that would correspond to mRNAs that contain only one of the three sliceable exons. The second minor band was approximately 370 bp in length and hybridized well with exon 5 and 6 probes and weakly with the exon 4 probe. Finally, most PC's contained a very small amount of a 534 bp fragment containing exons 4, 5 and 6. Although only a small number of pre-B cell and B-cell lines were surveyed in these preliminary experiments the foregoing data indicate that alternative splicing of CD45 may be restricted to the late stages of B cell development when B cells differentiate into plasma cells. To further explore this issue, we are studying additional pre-B cell lines including a previously described subset that are 6B2⁻ F-11⁺, additional B cell lines, cultured normal B cell precursors and resting and activated normal B cells.

Similar experiments to those described above were also performed with the myeloid progenitor lines M1, 32D, FDCP1 and FDCP2 as well as the macrophage lines 3G4, HAFTL-3A, J774 and P388D1. In all cases, the predominant CD45 mRNA species was 4.7 Kb, a size consistent with the deletion of exons 4, 5 and 6. PCR analysis confirmed that the predominant PCR fragment was 117 bp in length and did not hybridize with exon 4, 5 or 6 probes. Macrophages also had a minor PCR fragment of approximately 250 bp that hybridized most efficiently with the exon 4 probe. The myeloid progenitor lines had greater amounts of the 250 bp PCR fragment as well as a larger fragment of approximately 390 bp. Both fragments hybridized with exon 4, 5 and 6 probes and probably represent mixtures of fragments with exons 4, 5 and 6 alone or all combinations of two exons. To confirm that the 4.7 Kb CD45 mRNA is the predominant isoform in macrophages and does not contain exons 4, 5 or 6 we isolated CD45 clones from a cDNA library prepared from 3G4 cells. The library was screened with a full length cDNA isolated from T cells. Eighty three positive clones were analyzed with exon 3, 4 and 5 oligomer probes. No clones hybridized with the exon 4 and 5 probes but eight hybridized with the exon 3 probe. Three full length clones were introduced into pUC19 and the first six exons sequenced. The sequences were identical to each other and to the sequence published for exons 1, 2, 3, 7, 8 and 9. These data indicate that the deletion of exons 4, 5 and 6 occurs early in myeloid differentiation and is maintained in mature macrophages.

Studies of CD45 membrane proteins by immunoprecipitation confirmed that pre-B cells and B cells express a 220 KDa isoform and macrophages a 205 KDa isoform. In addition, we demonstrated that plasmacytomas express two different sized CD45 molecules, a predominant 180 KDa species and a minor species intermediate in size between the 180 and 220 KDa isoforms. A manuscript describing this work is in preparation.

Future research on this project will involve extensions of the studies outlined above. PCR analyses will be performed on normal pre-B cells, splenic B cells and peritoneal CD5⁺ B cells and macrophages. Polyclonally stimulated CD5⁺ and CD5⁻ B cells also will be examined to determine precisely when splicing occurs after cell activation. As mentioned above, chemical cross-linking techniques were used by others to demonstrate associations between CD45 and other molecules on the surface of T cells. We presently are attempting similar experiments to identify molecules that associate with CD45 in pre-B cells, B cells, plasmacytomas, myeloid progenitors and macrophages.

B. T Cell Abnormalities Associated with *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 expansion of a unique population of Ly-5(B220)⁺ CD4⁻ CD8⁻ (double negative; DN) T cells. In addition to these cells, *lpr* and *gld* mice also have elevated numbers of CD4⁺ and CD8⁺ T cells and a new category of CD4 dull⁺, Ly-5(B220)⁺ cells. Our long term goal is to determine the origin of the two B220⁺ subsets and understand how the four T cell populations accumulate, their relationship to one another and their involvement in promoting autoimmune disease. One possible mechanism by which T cells may accumulate is by the abnormal production of lymphokines that regulate their growth. To explore this possibility we examined C3H-*lpr* and -*gld* unfractionated LN cells and sorted Ly-5 (B220)⁺ DN T cells, CD4⁺ T cells and CD4 dull⁺ Ly-5 (B220)⁺ T cells for spontaneous and induced production of a spectrum of lymphokines. These studies revealed that DN T cells spontaneously neither transcribed mRNA for IFN- γ , TNF, GM-CSF, or interleukins 3, 4, 5 or 6 nor secreted detectable levels of these factors. Further, DN T cells treated with immobilized anti-TCR α/β or CD3 ϵ mAb failed to proliferate, secrete lymphokines or change in phenotype. Similarly, CD4 dull⁺, Ly-5(B220)⁺ T cells responded poorly to stimulation and did not produce significant levels of IFN- γ or TNF. Comparisons of surface antigen expression on the Ly-5(B220)⁺ T cell subsets revealed that CD4 dull⁺, Ly-5(B220)⁺ T cells closely resembled DN T cells in regard to size, expression of TCR α/β , CD3 ϵ , Ly-6C and CD44 suggesting that these two populations may be related. In contrast to the two Ly-5 (B220)⁺ T cell subsets, stimulated unfractionated *lpr* and *gld* LN cells proliferated strongly and secreted high levels of IFN- γ , TNF and GM-CSF and low levels of IL-3, -4 and -6. In primary cultures of normal T cells, IFN- γ is predominantly secreted by CD8⁺ T cells with production by CD4⁺ T cells dependent on prior activation. Surprisingly, stimulated *lpr* CD4⁺ Ly-5(B220)⁻ T cells produced very high levels of IFN- γ and higher than normal levels of TNF. Prior to stimulation, these cells also expressed higher levels of CD44 (an antigen expressed on activated and memory T cells) than unstimulated C3H-*+/+* CD4⁺ T cells. Together, these two findings are consistent with previous *in vivo* activation of a high proportion of *lpr* CD4⁺ T cells. The

increase in the absolute numbers of CD4⁺ T cells in lpr and gld lymphoid organs, as well as the potential for hypersecretion of lymphokines following stimulation, may result in local concentrations of growth factors that far exceed those in normal lymphoid tissues. Experiments to test this hypothesis are in progress.

In other studies, Ly-5(B220)⁺ DN T cells and various subsets of normal DN thymocytes were compared for cell surface antigen expression. The lpr and gld DN cells did not transcribe or express CD2 and phenotypically did not resemble freshly isolated thymic or peripheral CD2⁻DN T cell subsets. Preliminary experiments demonstrating that normal peripheral TCR α/β ⁺ DN T cells can express Ly-5(B220) after stimulation raise the possibility that lpr and gld DN T cells may arise from activated peripheral TCR α/β ⁺ DN T cells. An alternative source of the expanded DN T cell population may be mature T cells that no longer express CD2, CD4 or CD8. Manuscripts describing the cytokine secretion by T cell subsets and expression of CD2 on immature and mature DN T cells were published.

Most recently, we evaluated C3H-lpr and -gld CD8⁺ T cells for abnormalities. These studies revealed that like CD4⁺ T cells, CD8⁺ T cells also hypersecreted IL-2, IFN- γ and TNF following stimulation. Both CD4⁺ and CD8⁺ T cells have elevated levels of expression of the T cell activation marker, CD44, and decreased expression of Mel 14 and CD45R. These data are consistent with the activation in vivo of a high proportion of C3H-lpr and -gld CD4⁺ and CD8⁺ T cells. Ontogeny studies revealed that both CD4⁺ and CD8⁺ lpr T cells show evidence of activation by 8 weeks of age. In addition, we showed that B6-lpr and MRL-lpr mice also have polyclonally activated CD4⁺ and CD8⁺ T cells and hypersecrete inflammation-inducing cytokines following stimulation. A manuscript describing this work is in preparation.

Our future studies will focus on the influence of growth factors on lpr and gld T cell subsets, the derivation of B220⁺ DN T cells and CD4 dull⁺, B220⁺ T cells and the mechanisms governing the functional energy of some cells and the apparent hyperactivity of others.

Publications:

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SUMMARY STATEMENT
LABORATORY OF BIOCHEMISTRY

DCBDC, NCI

OCTOBER 1, 1990 TO SEPTEMBER 30, 1991

INTRODUCTION

Research in the Laboratory of Biochemistry continues to be diverse, reflecting the interests of fifteen independent groups, but during the past year the overlap in approaches and techniques has become more evident. Successful expression of the transcription factors involved in the regulation of gene expression has facilitated the study of these factors as proteins. Carl Wu and Dean Hamer have already made outstanding progress in this direction. Collaborative projects with investigators in the Chemical Biophysics Laboratory (NIADDK) have been initiated to determine the structures of these factors or of their active fragments by multidimensional NMR spectroscopy. There is also a growing interest in the potential regulation of these factors by protein phosphorylation.

Fruitful interchange among the members of the staff has increased significantly while the advantages of the laboratory's diversity of expertise have also become evident. It was therefore not unexpected that, when space became available because of the retirement of two members of the laboratory, the section chiefs recommended unanimously that two new members be recruited in lieu of an expansion of the existing sections. After a lengthy and diligent nation-wide search, two new Senior Staff Fellows were selected.

Charles Vinson, Ph.D., joined the laboratory in February 1991 and has already established an independent research program centered on the study of protein-DNA interactions. While he was a postdoctoral fellow in Steve McKnight's laboratory, Dr. Vinson contributed to the development of the general idea of a leucine zipper as a dimerization domain which led to a novel three dimensional model for the interaction of this class of protein with DNA. We believe that he will be a stimulating colleague and will strengthen the protein chemistry aspects of our research programs.

Dr. Mark Mortin will join us in October 1991. Dr. Mortin, a Drosophila geneticist interested in gene regulation and development, intends to combine biochemical and genetic approaches to study, the multisubunit enzyme, RNA polymerase II in Drosophila. Dr. Mortin's skills in Drosophila genetics will complement the current strengths of the laboratory and will be an asset for the several groups who plan to use genetic techniques in Drosophila as a strategy for the functional analysis of proteins in vivo.

These new appointments were made to replace Dr. Warren Evans who retired last year and Dr. Samuel Wilson who is leaving in December to accept a position of Director, The Sealy and Smith Center for Molecular Sciences, and Professor of Biochemistry at the University of Texas in Galveston. We were all sorry to learn of Sam Wilson's departure but share his enthusiasm about the opportunity he has been offered to expand his productive research program.

Last year's budget reduction, coupled with the increase of expenses due to inflation, made it difficult to meet all our needs. We are, however, fortunate that the additional funding requested to set up the Drosophila genetics facility has been approved and hope that next year's budget will allow us to replace outmoded equipment and to meet the demands of a rapidly changing and continuously improving technology. We hope to compensate for the reduction in personnel by a more selective recruitment process and will make every effort to retain our superb staff of young investigators. Despite these problems, the Laboratory of Biochemistry has remained in the forefront of biochemical research. The new appointments have strengthened our ability to study the complex network of factors controlling cell growth and differentiation.

REGULATION OF GENE EXPRESSION

The five groups studying the regulation of gene expression have all been successful in keeping up with the incredible pace of new discoveries in this area of research.

Dr. Wu's group has continued to study transcription factors regulating heat shock genes and the segmentation gene fushi tarazu, ftz, in Drosophila. Three transcription factors: Heat Shock Factor (HSF), FTZ-F1, and FTZ-F2 (now referred to as tramtrack) have been expressed in E. coli, and the recombinant proteins have been characterized biochemically and used for the preparation of antibodies. Immunostaining has revealed the subcellular distribution of the transcription factors and their pattern of expression in the developing embryo. This year's highlight was the successful cloning of a human heat shock transcription factor. Analysis of the cloned human HSF gene revealed the existence of a fourth, C-terminal leucine zipper motif in the Drosophila and human proteins, but which is absent in the yeast protein. This fourth zipper motif may be involved in the regulation of HSF DNA-binding activity, which is heat shock inducible in Drosophila and human, but not in yeast. The cloning of Drosophila and human HSFs opens the way for a comparative molecular dissection of the heat shock response. A second highlight is the immunolocalization of the HSF protein to chromosomal puff sites of the Drosophila polytene chromosomes. This localization takes the molecular analysis of the heat shock response full circle, back to the discovery of the heat shock phenomenon almost 30 years ago. In addition to the known heat shock loci, HSF is found at another 100 loci or so in the Drosophila genome. This finding may define new genes that are targeted for HSF control. A last highlight is the development of a highly efficient, in vitro, chromatin reconstitution system from early Drosophila embryos. Plasmid DNAs assembled into chromatin in the presence of exogenous histone H1 and crude embryo extract show nucleosome spacing indistinguishable from that found in native chromatin. This system will be very useful for future studies of the interaction of transcription factors with the chromatin template.

Dr. Hamer's group, in their continuing studies of gene regulation by metals, made the surprising discovery of an enzymatic reduction of copper. This process is highly regulated and appears to play a central role in the growth-promoting and repressing activities of this metal. The group has also obtained detailed structural information on a yeast metalloregulatory protein and identified new human and mouse metallothionein gene transcription factors.

While continuing to study the structure, regulation and mode of action of a group of genes involved in determining the muscle phenotype in vertebrates,

Dr. Paterson and his colleagues have initiated similar studies in Drosophila to take advantage of Drosophila genetics to facilitate identification of the physiological role of the factors encoded by these genes. Dr. Paterson and his colleagues have isolated two chicken myogenic factor genes, CMD1 and Cmg1, which are homologues of mouse MyoD and myogenin. During his sabbatical leave in Dr. Gehring's laboratory in Basel, Switzerland, Dr. Paterson also isolated a Drosophila homologue Dmyd. Each of the factors encoded by the members of this gene family shares a structural motif containing a basic region joined to a helix-loop-helix domain that is essential for function. The avian factors can induce the conversion of 10T1/2 fibroblasts to muscle cells, activate cotransfected muscle-specific promoter CAT constructs and bind to the MCK enhancer as a heterodimer with another helix-loop-helix transcription factor, E12. Despite its strong homology to mouse MyoD, the Drosophila factor cannot convert 10T1/2 cells, although it is efficiently expressed in mouse fibroblasts. It only weakly activates muscle-specific promoters and binds poorly to the MCK enhancer. Chimeric proteins that contain specific regions from the avian and Drosophila proteins are being used to identify regions responsible for the different activities. The regulation of these factors by protein phosphorylation is also being investigated and NMR studies are under way to elucidate the structure of the basic-helix-loop-helix motif of CMD1 and its associated transcription factors as monomers, heterodimers and complexes with the target DNA.

Dr. Vinson's research program is also focused on the class of DNA-binding proteins involved in muscle cell determination. He has demonstrated that one protein of this class binds to two cis elements in a cooperative fashion and he proposes that the interacting domain map to Helix II of the consensus sequence. He is presently testing an interesting model that accounts for the cooperative binding of this class of proteins to DNA.

The role of DNA methylation in the modulation of gene expression continues to be the major focus of interest of Dr. Kuff's laboratory. His group has been studying the regulation of an envelope-deficient mouse retrovirus, the intracisternal A-particle (IAP). IAPs are encoded by members of a multicopy family of endogenous proviral elements. IAP elements are transposable in the mouse genome. They are expressed in mouse embryos, some normal adult tissues, and many transformed mouse cells and represent a powerful model system for studying gene regulation. Expression requires demethylation of sequences within the IAP 5' LTR and a constellation of trans-acting factors. One such factor, designated EBP-80, has been purified and was shown to be composed of two polypeptides. Sequence analysis of tryptic peptides established that both components of EBP-80 are identical to those in a human protein, called Ku, previously identified as an autoantigen in general human disorders (autoimmune thyroiditis, disseminated lupus and others). The genetic basis for the selective demethylation and expression of IAP provirus is being investigated in normal mouse lymphocytes while the relevant transcription factors are being studied in nuclear extracts of transformed rodent and primate cells. Dr. Kuff and his colleagues have recently shown that hypomethylation and expression of IAP elements are early events in the development of thymus and LPS-stimulated splenic B cell tumors.

ORGANIZATION OF THE HUMAN GENOME

The organization of the human genome is the focus of interest of the groups led by Drs. Singer and McBride.

Dr. Singer and her colleagues continue to study the mechanism of LINE-1 transcription in the human genome. They have shown that both transcription and translation of the human transposable element LINE-1 (L1Hs) have unusual features. Cell-type-specific transcription of L1Hs mRNA is regulated by internal cis-acting motifs including an orientation-independent enhancer. Evidence is also accumulating for internal initiation of translation of both open reading frames.

Dr. McBride's expertise in chromosome mapping continues to attract many colleagues from NIH and elsewhere who seek his advice and collaboration. Complementary methods have been used to determine the chromosomal localization of a large number of cloned genes. During the past year 15 genes have been mapped by Southern analysis of DNAs from a panel of well characterized human-rodent somatic cell hybrids. Four genes have been localized by in situ hybridization with biotinylated DNA probes and four others were assigned by genetic linkage analysis with the 40 large CEPH kindreds. Clusters of 3-5 genes and anonymous DNA markers have been used to construct ordered linkage maps, each spanning about 20 cM, on chromosomes 14q11-q12, 22q11.2-q12, and 17q21-q22. A polymorphic trinucleotide repeat within the first intron of IL2RB has been used for linkage analysis in the CEPH pedigrees and IL2RB has been ordered within a cluster of five genes on 22q11.2-q12. This sequence and a polymorphic dinucleotide repeat flanking IL2RB have been used to establish linkage with NF2 in a 3-generation family segregating this gene. Progress is under way to isolate a series of highly polymorphic probes on chromosome 22 to be used to construct a high resolution genetic linkage map and physical map of human chromosome 22.

PROTEINS AND THE CONTROL OF CELLULAR PROCESSES

Several projects in the laboratory deal with the role of enzymes and other proteins in the regulation of cell function.

The Ca^{2+} -binding protein, calmodulin, plays a pivotal role in the Ca^{2+} regulation of cellular events such as cell division, secretion, motility, and the immune response. During the past year, Dr. Klee and her colleagues have concentrated their efforts on the Ca^{2+} and calmodulin stimulation of the protein phosphatase, calcineurin. Calcineurin is under a dual Ca^{2+} control mediated by an integral subunit of the enzyme, calcineurin B and by calmodulin. The high affinity Ca^{2+} sites of calcineurin B, fully occupied at resting levels of Ca^{2+} , are postulated to play a structural role. Occupancy of the Ca^{2+} sites of calmodulin needed for interaction with and activation of calcineurin requires the higher Ca^{2+} levels observed in stimulated cells. The Ca^{2+} dependence of calmodulin stimulation is highly cooperative and dependent on the concentration of free calmodulin. In related work, Dr. Frank Suprynowicz, with continuing support of Dr. Klee, has demonstrated that the concerted action of a protein kinase(s) and a phosphatase(s) is needed to inactivate, the mitosis-specific, cdc2 kinase and thereby to shut off mitosis. As a culmination of a two-year collaborative effort with Dr. Klee's group, Drs. Bax and Ikura (NIADKK) have

succeeded in determining the secondary structure of calmodulin in solution by multidimensional NMR spectroscopy.

The regulation of secretion by Ca^{2+} and GTP-binding proteins continues to be the focus of interest of Dr. Wagner and his colleagues. Having shown that norepinephrine secretion by chromaffin and PC12 cells is regulated by a Ca^{2+} -dependent phosphorylation, they are in the process of isolating cytosolic proteins involved in the secretory response. One of the proteins required for the Ca^{2+} -dependent secretion has been purified and is being characterized.

Shelby Berger and her colleagues are making steady progress in elucidating the role of prothymosin α in cell division. The function of this nuclear protein has been investigated using antisense oligomers directed against several regions of prothymosin α mRNA. These oligomers apparently bind to the mRNA and prevent accumulation of prothymosin α . Under these conditions, cells are unable to divide. Resumption of the cell cycle occurs only when the antisense oligomers are degraded intracellularly. This group also showed that two forms of prothymosin α mRNA arise by alternative splicing of one functional gene. The molecule formed according to consensus splice site selection contains an extra codon, and is rare. The common form of the mRNA, which violates splicing rules, occurs ubiquitously regardless of the type of tissue or the transformation history of cell lines. Support from the AIDS program has also allowed Dr. Berger's group to study the mechanism of action of reverse transcriptases. A complex composed of two ribonucleosides and one oxovanadium ion has been characterized as a potent inhibitor of the enzymes. With this inhibitor Dr. Berger's group provided strong evidence that a single DNA:RNA substrate binding site serves both the polymerase and the ribonuclease H functions of the enzyme.

Dr. Mage just finished a year's sabbatical leave in the laboratory of Dr. David Margulies, LI, NIAID, in order to obtain experience and training in molecular biology in the context of the MHC antigens. During this past year, he prepared a biologically active recombinant single chain Class I MHC molecule, in which the heavy chain and the light chain (beta 2-microglobulin) are covalently linked via a spacer peptide. The natural Class I MHC molecules consist of a heavy chain noncovalently linked to beta 2-microglobulin. The single chain molecule is potentially useful for structural studies, and will be used to study interactions of MHC molecules with antigenic peptides and T cell receptors.

In the past year Dr. Peterkofsky has concentrated on the identification of circulating inhibitors of collagen and proteoglycan synthesis that are induced in guinea pigs by vitamin C deficiency and fasting. Previous work suggested that the inhibitors consisted of two insulin-like growth factor-binding proteins (IGFBPs). One of the proteins, identified as IGFBP-1, is induced in scurvy. A second IGFBP, induced by these nutritional deficiencies, has tentatively been identified as IGFBP-2 based on its size (35 kDa) and ligand preference. In contrast, the level of the major circulating binding protein of normal guinea pig, identified as IGFBP-3, is not affected by the nutritional deficiencies.

DNA REPLICATION

Studies of Pl plasmid maintenance by the group of Drs. Yarmolinsky and Chatteraj focus on replication control, active partition and the lethal addiction of bacteria to a resident Pl plasmid. These studies have established

the DNA sequence features essential for RepA binding to its targets, provided evidence that RepA binding to the origin region is facilitated by heat shock proteins DnaJ, DnaK and GrpE and shown that this binding absorbs a positive superhelical turn of origin DNA. Other studies indicate that the Pl partitioning system can actively partition unreplicated DNA, but also reveal drastic context effects that can result in plasmid destabilization. A study of the effects of Pl plasmid loss reveals the existence of an addiction system that might act by a novel mechanism since the genes involved have no homologs of known function in other plasmids.

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 recombination in meiosis. They have shown that chromosome context is important in determining the site and number of these breaks. Their work suggests that break sites are determined primarily by elements of chromatin structure, rather than by recognition of particular sequences. In addition, DNA fragments that promote meiotic recombination in their vicinity have been cloned and are being characterized. These have been shown to contain elements that promote the formation of double-strand breaks in surrounding sequences.

Dr. Wilson and his colleagues have continued to take advantage of the bacterial overexpression of mammalian DNA polymerase β , Al hn RNP single strand-binding protein and HIV-1 reverse transcriptase to pursue their studies of DNA replication. Major findings include preliminary evidence that the control of β -polymerase expression is important for DNA repair and the detailed characterization of a transcription factor that regulates the β -polymerase gene by binding to a palindromic sequence with sequence homology to the CRE element of many viral and cellular promoters. With the continued strong support of the AIDS program, this group has made significant progress in the study of the structure-function relationship of HIV reverse transcriptase. The domain responsible for the interaction between the primer substrate and the enzyme has been identified by UV-crosslinking of the oligo d(T)-enzyme complex and subsequent sequencing of V8 protease peptides. Information on the properties of this substrate binding domain will be used to design more specific inhibitors of HIV-1 reverse transcriptase.

Project DescriptionMajor Findings:A. Transacting Cellular Factors for IAP Expression (Dr. M. Falzon)

Transcription of endogenous IAP proviral elements is determined by cellular factors acting in trans on specific regulatory motifs in the IAP 5' LTR. At least five such nucleotide motifs have been identified by sequence analysis and/or protein-binding studies of cloned IAP LTRs. Our previous reports have described the isolation of a transcription factor, which we designated EBP-80, that bound tightly to a sequence motif with homology to the enhancer core sequences in SV40, polyoma and the KiMSV LTR. Similar preparations, composed of two proteins with apparent sizes of 70 and 85kD, were obtained from both human 293 cells and mouse myeloma. During the past year, Dr. Falzon prepared a large quantity of highly purified human EBP-80, and was able to separate the two components in amounts sufficient for micro amino acid sequencing. Sequence was obtained for two tryptic peptides from each protein. A search of the gene bank identified both proteins unequivocally as components of a previously known protein preparation, designated Ku, that was originally identified as an autoantigen in several human disease states (autoimmune thyroiditis, disseminated lupus, etc). cDNAs for both proteins have been cloned from HeLa cells and sequenced in other laboratories. Fractions with very similar or identical physical properties have been identified as DNA-binding proteins and/or transcription factors for several other genes. Ku and related proteins are known to bind to the ends of linear duplex DNA molecules but not to closed circular DNAs. A role for the proteins in recombination or DNA repair has been suggested but not established experimentally.

We have confirmed the end-binding properties of EBP-80 and its failure to bind to closed circular DNA under the usual assay conditions. Since these properties are inconsistent with the fact that EBP-80 is an effective enhancer of in vitro transcription from closed circular templates, we have begun an in depth analysis to clarify the mechanism of action of this factor. Dr. Falzon showed first that the plasmid templates used for transcription remained in closed circular form during the in vitro incubation, and secondly that EBP-80 will bind to closed circular template under the buffer conditions used in the transcription reaction. Quantitative filter-binding assays show that EBP-80 binds to DNA ends with high affinity (10-20pM). However it also binds tightly to linear duplex DNA molecules in which the strand ends are connected by short single-stranded loops, i.e., to molecules that contain no interruptions of the phosphate-sugar backbones. The likely binding sites in these constructs appear to be regions of transition from double- to single-stranded states. This unusual property of EBP-80 suggests that it may bind to regions of DNA unwinding or to palindromic stem-loop projections, both configurations implicated in transcriptional regulation.

We have previously reported evidence that the known inhibitory effect of DNA methylation on promotion from IAP LTRs is mediated in part through an effect on EBP-80 binding (see Annual Report 1989-1990). Since the relevant mCpG pair for this effect lies within an alternating purine/pyrimidine tract that can assume Z-DNA configuration, and since C-methylation is known to favor the B-to-Z

transition, we also are considering the possibility that EBP-80 binding may be sensitive to this and other local variations in DNA conformation. Studies on EBP-80 binding and function will be continued.

B. Selective Activation of IAP Proviral Elements in Mouse Lymphocytes
(J. Mietz).

As described in last year's Annual Report of this project, IAP-related cDNAs isolated from both thymus and LPS-activated splenic B-cells of BALB/c mice were derived primarily from transcripts of a few very closely related IAP elements. Small sequence differences among the cDNAs has permitted identification of three families of expressed elements (termed LS elements) containing in aggregate 13 active IAP genes that account for at least 70% of the IAP transcripts in both types of lymphocytes. The U3 and R regions of these 13 elements show distinctive nucleotide sequence differences from those of all previously reported IAP elements except one cDNA clone from normal BALB/c mouse brain. Hybridization of Northern blots or RNA dot blots with oligonucleotide probes specific for the three families of LS elements in BALB/c mice reveals that representatives of all three are expressed in thymus of other inbred mouse strains. Restriction enzyme digests of genomic DNAs were fractionated by high resolution gel electrophoresis in one dimension and hybridized in the gels with the three LS family-specific oligonucleotide probes. The band patterns indicated some 10 to 15 members of each family in BALB/c DNA. A similar number of bands were found in digests of DNA from mice of 7 other inbred strains. Some bands were shared among several or all strains, but extensive strain-related polymorphisms were observed.

As mentioned in section A above, IAP expression seems to require demethylation at certain mCpG sites in the 5' LTRs. We have devised a 2-dimensional agarose gel electrophoretic method that permits detection of individual LTR-demethylated IAP genomic elements against the background of methylated copies. Hybridization of such 2-D gels with the LS-specific oligonucleotides showed that a limited number of elements within each family was demethylated in DNAs from thymus, activated B-cells and several other tissues. Thus a certain fraction of the total family members appeared to be constitutively activated for transcription in these normal cells. Many more IAP elements of all types were found to be demethylated in IAP-rich established myeloma cells. Demethylation of IAP sequences was also extensive in a series of primary myelomas. Mesenteric granulomas from pristane-treated mice early in the process of B-cell tumorigenesis were sectioned and immunostained with antibodies against the IAP structural protein p73. Large IAP-positive cells were detected in some but not all foci of proliferating plasma cells. Together, these observations suggest that accentuated demethylation and expression of endogenous IAP elements may be a very early earmark of B-cell neoplasia.

The findings outlined in the first paragraph of this section represent the first demonstration that the extensive family of IAP proviral elements, some 1000 per haploid genome, can be resolved on the basis of small characteristic sequence differences into small subsets of highly related members. They raise interesting problems about the coordinate regulation of these dispersed but related elements. In addition, they provide a large set of potential specific markers for analysis of the mouse genome.

Publications:

Falzon M, Kuff EL. A variant binding sequence for transcription factor EBP-80 confers increased promoter activity on a retroviral LTR, J Biol Chem 1990;265:13083-90.

Kuff EL. Intracisternal A-particles in mouse neoplasia, Cancer Cells 1990;2:398-400.

Falzon M, Kuff EL. Binding of the transcription factor EBP-80 mediates the methylation response of an intracisternal A-particle LTR promoter, Mol Cell Biol 1991;11:117-25.

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

PROJECT NUMBER

Z01 CB 00945-18 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

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

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

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:

3

PROFESSIONAL:

2

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.)

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 inhibited DNA synthesis in 3T3 cells and collagen and proteoglycan synthesis in cultured primary chondrocytes and adult, but not fetal, human skin fibroblasts. The presence of the inhibitor in fasted and scorbutic sera was correlated with an increase in low molecular weight insulin-like growth factor (IGF)-binding proteins (30-50 kDa) that can inhibit binding of IGF-I to its receptor on 3T3 cells and human fibroblasts. Our recent studies are concerned with identifying the IGF binding proteins in guinea pig sera. One of the IGF-BPs that is increased in scorbutic and fasted guinea pig serum has been identified as IGF-BP-1, a 30-kDa protein, by using ligand blotting, Western blotting and immunoprecipitation. The second binding protein that is increased has been tentatively identified as IGF-BP-2 based on its ligand binding specificity and size (35 kDa) observed on ligand blots. IGF-BP-3 could not be observed by the previous methods that depended on the presence of a free IGF binding site, since it exists in serum as a complex with the IGFs. It can be detected on ligand blots since complexes are dissociated during SDS-PAGE. We identified a doublet of 41 and 44-kDa binding proteins as glycosylated forms of BP-3 and their levels were unchanged during scurvy and fasting. Characterization of the induced proteins and studies to confirm their function as inhibitors of IGF-I function are continuing.

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) in this regulation.

Major Findings:

Regulation of Collagen and Proteoglycan Synthesis During Scurvy and Fasting

Our previous results suggested that ascorbate-deficient and fasted, but ascorbate-supplemented, guinea pigs are equivalent with respect to the mechanisms by which collagen and cartilage proteoglycan synthesis are decreased. Furthermore, sera from these animals could transmit the defects in extracellular matrix synthesis to cultured chick embryo chondrocytes and human fibroblasts, in the presence of ascorbate, and this effect resulted from the presence of an inhibitor. These sera also inhibited the stimulation of DNA synthesis in quiescent 3T3 cells by normal guinea pig serum. The inhibition of these processes in both cell types was reversed by IGF-I. The ability of IGF-I to reverse inhibition of various cellular functions by fasted and scorbutic sera suggested that the inhibitor might be an IGF-I binding protein (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. On gel filtration, inhibition of binding of IGF-I to its receptor on 3T3 cells coincided with IGFBP activity.

A. Identification of IGFBPs in Guinea Pig Sera

In our recent studies, we have used ligand blotting to analyze and identify the IGFBPs in guinea pig sera. In this procedure, sera is electrophoresed on SDS-PAGE, blotted to a membrane and incubated with the radioactive ligand. Since complexes of IGF bound to binding proteins are dissociated during SDS-PAGE, this procedure measures the total IGFBPs in the sera, including IGFBP-3 which exists in sera almost exclusively as a complex with IGF. Three major IGF binding proteins were identified based on comparison to IGFBPs that have been characterized in other species:

IGFBP-3. In other species, this binding protein is the only one of the five recognized IGFBPs that is N-glycosylated and binds to concanavalin A. It was identified in guinea pig sera using ligand blotting as doublet bands corresponding to proteins of approximately 41 and 44 kDa. These proteins were converted to a single 38-kDa band after treatment with the enzyme N-glycanase, which cleaves off oligosaccharide units linked to N-asparagine. Further confirmation that the doublet band was glycosylated BP-3 was the fact that they were bound to a concanavalin A column and could be eluted with α -methyl mannoside. There was essentially no change in the level of this binding protein during scurvy or fasting.

IGFBP-1. A protein that appeared as a 30-kDa band in ligand blots was identified as IGFBP-1 by Western blotting of the dissociated protein, using

an antiserum against human IGFBP-1 obtained from Dr. David Clemmons. Immunoprecipitation of BP-1 cross-linked to [¹²⁵I]IGF-I showed that this protein corresponds to the 38-kDa cross-linked binding protein observed on SDS-PAGE that was increased in sera during scurvy and fasting. The 30-kDa binding protein observed on S200 gel columns that increased in scorbutic and fasted sera also appears to be BP-1.

IGFBP-2. Circumstantial evidence suggests that a 35-kDa IGFBP observed by ligand blotting to be increased to a greater extent in scurvy that in fasting is IGFBP-2. This protein corresponds to the 42-44-kDa cross-linked species that we previously observed to be increased differentially during these nutritional deficiencies. Evidence for this conclusion includes the correspondence in size with BP-2 secreted by MDBK cells, the bovine form of BP-2 which has been purified and sequenced. In addition, BP-2 from other species has greater affinity for IGF-II than for IGF-I and the 42-44-kDa cross-linked binding protein shows similar behavior, in contrast to BP-3 and BP-1 which have approximately equal affinity for the two IGFs. Antibody for IGFBP-2 is not available to confirm the identification, but since the reported sequences for BP-2 from several species show a high degree of identity, we plan to prepare antibody from peptides. We have had peptides prepared that correspond to several conserved sequences and will prepare antigen by coupling the peptides to keyhole limpet hemocyanin.

B. Differential Inhibition of Collagen Synthesis in Cultured Human Fibroblasts From Old and Young Donors by Scorbutic and Fasted Guinea Pig Sera

In setting up an assay to measure the inhibitor of collagen and proteoglycan synthesis in sera from nutritionally deficient guinea pigs, we found that human fibroblasts from adult donors were sensitive to the inhibitor as opposed to fibroblasts from fetal donors. In addition, we observed that the rate of collagen synthesis in the adult fibroblasts was much lower than in the fetal fibroblasts. It is known that there are changes in extracellular matrix formation as skin ages in humans and since the changes in collagen synthesis that we observed could be related to this phenomenon, we examined the effect in greater detail. The following observations were made:

- 1) The extent of the decrease in both collagen synthesis and the levels of mRNA for collagen types I and III, the major collagen types produced by human fibroblasts, were related to the age of the donor.
- 2) Continuous subculturing of fetal fibroblasts resulted in a decrease in their rate of collagen synthesis and expression of mRNA for collagen types I and III. By passage 30, the cells resembled fibroblasts from aged donors with respect to these functions as well as growth rates.

These results support the hypothesis that in vivo and in vitro aging produces similar changes in human fibroblasts and suggest that the effects of aging on the extracellular matrix in skin may be due to decreased collagen synthesis by fibroblasts.

Publications:

Peterkofsky B, Palka J, Wilson S, Takeda K, Shah V. Elevated activity of low molecular weight insulin-like growth factor-binding proteins in sera of vitamin C-deficient and fasted guinea pigs, *Endocrinology* 1991;128:1769-79.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05202-24 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
B. Watson	Graduate Student, Howard University	LB	NCI
W. Fibison	Guest Researcher	LB	NCI
J. Clark	Laboratory Worker	LB	NCI

COOPERATING UNITS (if any) NICHD: W. Leonard, R. Klausner, I. Owens. NIAMSD: P. Steinert. NCI: A. Fornace, C. Klee, E. Kuff, K. Kelly, P. Steeg, D. Parry. NHLBI: R. Adelstein. NIDDK: A. Burns. NINDS: R. Eldridge. NIAID: V. Siebenlist. R. Pirtle, U. Texas, E. Strehler, Zurich. R. Iozzo, T. Jefferson School of Medicine

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

B

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

Mapping of cloned human genes is performed in this laboratory by three complementary methods including Southern analysis of DNAs from a panel of well-characterized human-rodent somatic cell hybrids, *in situ* hybridization of metaphase spreads with biotinylated DNA probes, and genetic linkage analysis in the 40 large C.E.P.H. pedigrees. During the past year, about 15 additional genes have been chromosomally localized using the panel of somatic cell hybrid DNAs. Four genes were also regionally localized by *in situ* hybridization and another four genes by genetic linkage analysis. Two functional genes (NM23H1 and NM23H2) in a multigene family of tumor suppressor genes were found to be tandemly linked (head to tail) on chromosome 17q21-q22 and closely linked to a cluster of genes (HOX2, NGFR, and COL1A1) which are known to be physically linked within a one megabase region. Surprisingly, a sequence which cross-hybridizes strongly with the NM23H1 probe is located on a different chromosome (i.e. 16). Ordered linkage maps of genes on 14q11-q12, 22q11.2-q12, and 17q21-q22 have been constructed from our linkage data. An (AAT)₁₇ trinucleotide repeat sequence within the first intron of the interleukin 2 receptor γ -chain gene (IL2RB) has been used for genetic linkage analysis of IL2RB by sequencing gel size analysis of this highly polymorphic sequence after PCR amplification using oligonucleotide primers flanking this sequence. IL2RB has served as an anchor locus to order five genes on this region of chromosome 22 as well as several flanking anonymous DNA markers and genes. More importantly, in collaboration with R. Eldridge and D. Parry, the polymorphic trinucleotide repeat and a (TG)₂₆ dinucleotide repeat in the 5' untranslated region of IL2RB were used to localize the NF2 (neurofibromatosis 2) gene on chromosome 22 in a 3-generation family segregating this gene. Additional studies are in progress to further localize this gene. A group of 35 plaques containing a trinucleotide repeat have been isolated from a chromosome 22 library and they are being used to construct a high resolution genetic linkage map and physical map of chromosome 22.

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, and 3) developing a map of the human genome and identifying specific genes or gene alterations involved in hereditary diseases.

Major Findings:

A panel of human-rodent somatic cell hybrids previously isolated and characterized in this laboratory continues to be used for chromosomal mapping of cloned human genes in collaboration with investigators at NIH and elsewhere. Some of the genes mapped by this method (and collaborators) during the past year have included a tyrosine phosphatase and the gene for a ras-related protein (K. Kelly, NCI), transglutaminase K (P. Steinert, NIAMSD), two different transcriptional activators in T cells (U. Siebenlist, NIAID), another gene (IRE-BP2) for a family of iron responsive element binding proteins (R. Klausner, NICHD), a gene for the 80K Ku autoantigen (E. Kuff), heparin sulfate proteoglycan (HSPG) and clathrin genes (R.V. Iozzo, Jefferson School of Medicine), NM23H2 (another member of a family of genes implicated in tumorigenesis and metastasis) (P. Steeg, NCI), PMCA2 (a third member of the plasma membrane Ca ATPase family), and calcineurin A alpha (C. Klee). Genes for a uridine monophosphate glucuronosyl transferase family (I. Owens, NICHD), loricrin (P. Steinert), and two genes involved in DNA repair (A. Fornace, NCI) have been regionally localized by in situ hybridization with biotinylated DNA probes. The tyrosine phosphatase gene (K. Kelly), HSPG (R. Iozzo), NM23H1 (P. Steeg), and two lymphokines designated 464 and 744 (U. Siebenlist) have been regionally localized by genetic linkage analysis in the 40 large C.E.P.H. pedigrees using RFLPs as markers or by PCR amplification of microsatellite sequences in the case of the interleukin 2 receptor beta chain gene (W. Leonard).

These complementary methods have provided several interesting results including evidence for tandem duplication of genes in some of the multigene families examined whereas others are widely dispersed on different chromosomes and some exhibit a mixture of both dispersion and tandem duplication. Linkage analysis now localizes the tumor suppressor gene, NM23H1, to chromosome 17q21-q22 in close linkage with HOX2, NGFR, and COL1A1 genes. The same probe identifies a closely related sequence (most likely a retro-pseudogene) on chromosome 16, whereas another functional gene (NM23H2) in this same multigene family is located on chromosome 17 in a head-to-tail orientation with the NM23H1 gene. These two functional genes are separated by less than 7 kb since cDNA probes for both genes identify a common polymorphic BglII fragment of this size but NM23H1 and NM23H2 detect only 2 kb and 5 kb allelic bands, respectively. We previously demonstrated that a region on chromosome 17 containing two genes (464 and 744) (U. Siebenlist) for lymphokines separated by about 8 kb was reduplicated on most copies of this chromosome in normal individuals, and RFLPs were detected at each site. Genetic linkage analysis in the C.E.P.H. pedigrees now demonstrates that

this locus is in close proximity to the chromosome 17 centromere and the NF1 locus. This locus is reduplicated on chromosome 17 in the majority of cases (i.e. 70%) and it appears to involve an amplification unit of about 1-2 megabases based upon the presence of two recombinants between these two sites. This reduplication and a third less frequent (i.e. 10%) copy are genetically stable and segregate in a Mendelian manner.

An (AAT)₁₇ highly polymorphic (PIC=0.70) trinucleotide sequence within the first intron of the interleukin 2 receptor 8-chain gene (IL2RB) found by W. Leonard (NICHD) has been used for linkage analysis. All members of the C.E.P.H. families were examined by PCR amplification of DNAs using oligonucleotide primers flanking the repeat and then determination of fragment lengths by denaturing polyacrylamide gel electrophoresis and autoradiography. Two point and multipoint linkage analysis with other genes mapped to chromosome 22 in this laboratory permitted construction of an ordered linkage map of this 22q11.2-q12 region consisting of (centromere)---MYH9 (non-muscle myosin HCA)-IL2RB-CYP2D-PDGFB(Sis)-thyroid autoantigen---(telomere). Two additional genes and three anonymous DNA markers were also ordered and linked to this gene cluster. The entire region spanned is about 50 centimorgans (cM), and the high polymorphic information content of these gene markers (especially IL2RB) made this analysis possible. This provides a high resolution map for the middle of chromosome 22q which is linked to another set of four anonymous DNA markers spanning about 20 cM in 22q11.1 ordered by Wendy Fibison in this laboratory.

In collaboration with Drs. Roswell Eldridge and Dilys Parry of the Inter-institute Genetics group, these polymorphic markers on chromosome 22 have been used to localize the NF2 (neurofibromatosis type 2) gene to this chromosome in a large three-generation family carrying this autosomal dominant gene. An additional polymorphic (TG)₂₆ dinucleotide repeat from the 5' untranslated region of IL2RB was also used for this analysis employing the same methods described for the (AAT)₁₇ sequence. Briefly, the results indicate that NF2 is linked to IL2RB with a significant Lod score (Z) of 3.5 at $\theta_{max}=0.083$. Significant evidence for linkage (i.e. $Z \geq 3.0$) could only be demonstrated for the highly informative IL2RB marker. The results also indicate that the NF2 gene is telomeric to an anonymous DNA marker, D22S1, which is located 15-20 cM centromeric to IL2RB. Multipoint analyses are in progress, and additional multiallelic probes are being isolated to allow higher resolution mapping of the NF2 locus and cloning of the gene.

Microsatellites (i.e. oligonucleotide repeats of di-, tri-, tetra-nucleotides, etc.) can provide highly informative probes for constructing a high resolution genetic linkage map and physical map of chromosomes, and chromosome 22 has been chosen for this type analysis. Ten oligonucleotide probes have been synthesized which are sufficient to detect all 60 potential trinucleotide repeat sequences. A complete digest (EcoRI) chromosome 22 recombinant λ phage library (LA22NS03) has been screened with one of these ³²P-end-labeled probes and 100 positive plaques were detected in 15×10^3 plaques screened (i.e. one repetitive sequence per 600 kb). Thirty-five positive plaques are currently undergoing plaque purification. Minipreps of the recombinants will be used for PCR amplification, assignment to specific regions of chromosome 22 by Southern analysis of hybrid cell DNAs containing defined portions of this chromosome, and subcloning of regions flanking the trinucleotide repeats. The sequence of nucleotides

flanking these repeats will be determined to permit synthesis of specific oligonucleotide primers which can be used subsequently for genetic linkage and physical mapping studies.

Publications:

Oerter KE, Munson PJ, McBride OW, Rodbard D. Computerized estimation of size of nucleic acid fragments using the 4-parameter logistic model, *Analyt Biochem* 1990;189:235-43.

Mock B, Krall M, Kozak CA, Nesbitt M, McBride OW, Van Snick J. A T cell growth factor gene, P40, maps to mouse chromosome 13 and human chromosome 5, *Immunogenetics* 1990;31:265-70.

Gan S-Q, McBride OW, Idler WW, Markova N, Steinert PM. Organization, structure and polymorphisms of the human profilaggrin gene, *Biochemistry* 1990;29:9432-40.

Akamizu T, Ikuyama S, Saji M, Kosugi S, Kozak C, McBride OW, Kohn LD. Cloning of the FRTL-5 rat thyroid Thyrotropin receptor: chromosomal assignment and down regulation by thyrotropin and agents which increase cAMP levels including thyroid stimulating autoantibodies, *Proc Natl Acad Sci USA* 1990;87:5677-81.

Irving SG, Zipfel PF, Balke J, McBride OW, Morton CC, Burd PR, Siebenlist U, Kelly K. Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q, *Nucleic Acids Res* 1990;18:3261-70.

McBride OW, Kozak CA, Wilson SH. Mapping of the gene for DNA polymerase B to mouse chromosome 8, *Cytogenet Cell Genet* 1990;53:108-11.

Rouault TA, Tang CK, Kaptain S, Burgess WH, Haile DJ, Samaniego F, McBride OW, Harford JB, Klausner RD. Cloning of the cDNA encoding an RNA regulatory protein-the human iron responsive element binding protein, *Proc Natl Acad Sci USA* 1990;87:7958-62.

Solomon SD, Geisterfer-Lowrance A, Vosberg H-P, Hiller G, Jarcho JA, Morton CC, McBride OW, Mitchell AL, Bale AE, McKenna W, Seidman JG, Seidman CE. A locus for familial hypertrophic cardiomyopathy is closely linked to the cardiac myosin heavy chain genes, CRI-L436, and CRI-L329 on chromosome 14 at q11-q12, *Am J Hum Genet* 1990;47:389-94.

Srivastava M, McBride OW, Fleming PJ, Burns AL. Genomic organization and chromosomal localization of the human nucleolin gene, *J Biol Chem* 1990;265:14922-31.

Young MF, Kerr JM, Termine JD, Wewer U, Wang MG, McBride OW, Fisher LW. cDNA cloning, mRNA distribution and heterogeneity, chromosomal localization, and RFLP analysis of human osteopontin, *Genomics* 1990;7:491-502.

McBride OW, Fisher LW, Young MF. Localization of PGI (Biglycan, BGN) and PGII (Decorin, DCN, PG-40) genes on human chromosomes Xq13-qter and 12q, respectively, *Genomics* 1990;6:219-25.

Papathanasiou MA, Kerr NCK, Robbins JH, McBride OW, Alamo I Jr, Barrett SF, Hickson ID, Fornace AJ Jr. Induction by ionizing radiation of the gadd45 gene in cultured human cells: lack of mediation by protein kinase C, *Mol Cell Biol* 1991;11:1009-16.

Olson S, Wang MG, Carafoli E, Strehler EE, McBride OW. Localization of two genes encoding plasma membrane Ca^{2+} -transportion ATPases to human chromosomes 1q25-32 and 12q21-23, *Genomics* 1991;4:629-41.

Simons M, Wang M, McBride OW, Kawamoto S, Yamakawa K, Gdula D, Adelstein RS, Weir L. Human nonmuscle myosin heavy chains are encoded by two genes located on different chromosomes, *Circ Res* 1991;69:1-10.

Peterson DD, McKinney CE, Ikeya K, Smith HH, Bale AE, McBride OW, Nebert DW. Human CYP1A1 gene: cosegregation of the enzyme inducibility phenotype and an RFLP, *Am J Hum Genet* 1991;48:720-5.

Mitchell A, Bale AE, Wang-ge M, Yi HF, White R, Pirtle RM, McBride OM. Localization of a DNA segment encompassing four tRNA genes to human chromosome 14q11 and its use as an anchor locus for linkage analysis, *Genomics*, in press.

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

PROJECT NUMBER

Z01 CB 05203-23 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:

2.50

PROFESSIONAL:

2.50

OTHER:

-

CHECK APPROPRIATE BOX(ES)

(a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews AIDS research: 50%

B

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

Our laboratory has a long standing interest in development of immunochemical methods for cell separation, for studying receptor-mediated activation of T cells, and in the role of T cells in tumor rejection. With respect to immune activation, we are particularly interested in developing methods for the direct measurement of biologically important but relatively low affinity immune interactions. Such methods include the use of semisynthetic multivalent macromolecular reagents ("polygens"), and recombinant multimeric protein domains. These constructs, which increase the effective valence of protein epitopes, may help to make it possible to do direct measurements of such interactions as between the T cell receptor and peptide-pulsed MHC antigens.

During this year, I have been doing an intramural sabbatical in the laboratory of Dr. David Margulies, LI, NIAID, in order to expand my capabilities by obtaining experience and training in molecular biology, in the context of Class I MHC molecules. In my sabbatical work, I have prepared a biologically active recombinant single chain Class I MHC molecule, potentially useful for structural studies, and for studying interactions of MHC molecules with antigenic peptides and T cell receptors.

Project Description

Objectives:

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

Major Findings:

- A. A recombinant gene encoding a single chain Class I MHC molecule has been prepared and expressed in mammalian cells. The natural class I molecules, which are expressed on the surfaces of most vertebrate cells, consist of a heavy chain noncovalently associated with beta 2 microglobulin, and with peptides derived from endogenously synthesized proteins. When cells are virally infected, for example with HIV1, the Class I MHC molecules present viral peptides to the antigen specific receptors of T cells, resulting under some circumstances in the killing of the infected cells. In the recombinant molecule, the beta 2 microglobulin carboxy terminal is linked via a spacer peptide to the amino terminal of a heavy chain known to present a peptide from the gp120 envelope protein of HIV1. It was prepared by linking the gene for beta 2 microglobulin, via oligonucleotide sequences encoding the spacer peptide, to a gene for a soluble Class I heavy chain. The resultant construct, ligated into a vector with a beta actin promoter, was transfected into L cells, which secrete the soluble recombinant Class I MHC molecule into the culture medium.
- B. The recombinant molecule has been shown to be correctly folded, by reaction with conformationally sensitive antibodies to the heavy chain, in ELISA assay and by immunoprecipitation.
- C. The recombinant molecule is biologically active, as shown by its ability to stimulate an alloreactive T hybridoma, in a manner comparable to the natural two chain Class I MHC molecule.
- D. A second recombinant gene for a single chain Class I MHC molecule has been prepared, encoding a molecule with the beta 2 microglobulin gene linked to the carboxyl terminal of the heavy chain. Comparison of the two recombinant proteins may yield information about the order of folding, assembly, peptide binding, and extracellular expression of Class I MHC molecules.

Publication:

Mage MG. Fractionation of T cells and B cells by panning techniques. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. Current protocols in immunology. New York: John Wiley and Sons, 1991;3.5.1.-3.5.6.

Patent:

Mage M, Nardelli B, McHugh L. Polyacrylamide - Streptavidin: A novel reagent for simplified construction of soluble multivalent macromolecular conjugates.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05214-20 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

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

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

S. H. Wilson	Chief, Nucleic Acid Enzymology Section	LB	NCI
S. Widen	IRTA Fellow	LB	NCI
E. Englander	Visiting Fellow	LB	NCI
A. Kumar	Expert	LB	NCI

COOPERATING UNITS (if any)

D. Lowy, NCI; T. Jeang, NIH; A. Fornace, NCI; K. Williams, Yale; R. Karpel, Univ. of Maryland

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

We continued biochemical studies of mammalian DNA replication proteins. cDNAs for rat and human β -polymerase have been subcloned in expression vectors, and the proteins have been overproduced in *E. coli*, purified in mg quantities, and sequenced. Structure-function aspects and *in vitro* DNA repair activities of the recombinant enzymes are being studied. Genomic DNA spanning the gene for human β -polymerase was further characterized. The promoter region was studied by detailed deletion and site-directed mutagenesis using a transient expression system for assay. In other work, we purified and characterized a transcription factor for the human β -polymerase gene. *In vitro* transcription studies of the gene are under way, as well as physical biochemical studies of the sequence-specific binding between the promoter and the purified factor. Studies of cell lines carrying inducible β -polymerase mRNA antisense transcripts revealed that down regulation of β -polymerase expression is associated with a block in DNA repair after nitrogen mustard treatment of cells.

Project DescriptionObjectives:

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

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

Major Findings:A. Structure and Function of Mammalian DNA Polymerases

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

1. DNA β -Polymerase

Background:

DNA polymerase β (β -pol) is a housekeeping enzyme considered to be involved in gap-filling synthesis during DNA repair in vertebrate cells. In many cell lines, β -pol mRNA levels are typical of other cases of low-abundance, "housekeeping gene" mRNAs. However, abundant expression of β -pol mRNA and protein is found in some cell lines and tissues. For example, survey experiments of rodent tissues reveal that liver contains slightly less β -pol mRNA than most other tissues, whereas testes contain much more than other tissues and perhaps 50-fold more than liver; some human cell lines, such as teratocarcinoma NTeraD2 have about a 5-fold higher level of the β -pol transcript than reference cell lines such as fibroblast AG1522. Further, induction of β -pol mRNA level (4-fold) is seen within four hours after treatment of CHO cells with the single-base alkylating agents MNNG, MMS, or AAF; by contrast, induction is not seen with other DNA damaging agents.

Findings:

- a. To ascertain whether differential mRNA expression could be studied at the level of the β -pol promoter, fusion gene constructs were prepared with the human β -pol core promoter (~100bp) linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. It was found that β -pol promoter activity in transient expression experiments is much higher in MNNG damaged CHO cells than in untreated cells and, in addition, that the β -pol promoter can be strongly activated by transcriptional control proteins such as ElA/ElB and Harvey ras.

- b. Studies of mechanisms of regulation of the human β -polymerase (β -pol) promoter were continued. The purified ATF/CREB family bovine testes sequence-specific DNA binding protein (described in the last report) that binds very tightly to the β -pol promoter and increases transcription in an in vitro system was further characterized. Using a series of selectively mutagenized promoters, the binding specificity of the purified protein was found to depend upon residues both flanking and within the 10 base-pair palindromic sequence (GTGACGTCAC) which is in the center of the ~22 base-pair binding site. The free energy of in vitro binding to each of the mutated promoters is being compared with the in vivo promoter activity in 293 cells.

Efforts to obtain cDNA clones for this binding protein are under way. But, in the meantime, biochemical properties of the purified protein were further studied. It was found that the protein is phosphorylated in vivo and that it is isolated as a phosphoprotein. Dephosphorylation of the protein in vitro with phosphatase led to a decrease in the stability of the protein: β -pol promoter complex, suggesting that phosphorylation of the protein can play a regulatory role in the metabolism of this particular ATF/CREB site binding protein.

- c. Characterization of the domain structure of mammalian DNA polymerase beta was conducted. Large-scale overproduction of the rat protein in Escherichia coli was achieved, and the purified recombinant protein was verified by sequencing using tryptic peptides. As revealed by controlled proteolysis experiments, the protein is organized in two protease-resistant segments linked by a ~15-residue protease sensitive region. One of the protease-resistant segments represents the NH₂-terminal 20% of the protein, and this NH₂-terminal domain (of about 75 residues) has binding affinity for ssDNA (i.e., template) approximately equal to that of the intact protein. The other protease-resistant segment, representing the COOH terminal domain of ~250 residues, does not bind to ssDNA, but has substantial DNA polymerase activity. This fragment is able to bind double-stranded DNA, however. The results are consistent with the idea that the NH₂-terminal domain is responsible for template DNA binding activity of the intact protein, whereas the C-terminal is responsible for polymerization and primer binding. A 16 kDa cyanogen bromide fragment spanning from residue 18 to the center of the protein is able to bind both ssDNA and dsDNA. This fragment may represent the portion of the enzymatic active site involved in template-primer binding.
- d. DNA polymerase beta (β -pol) and its mRNA are maintained at constitutive level during the cell cycle and during stages of cell growth in culture. To study biological consequences of variations in the level of this DNA repair enzyme and/or its mRNA, we prepared an expression vector in which cDNA for human β -pol is inserted under the control of a metallothionein promoter (pMT) in the antisense orientation, and this vector then was used for stable transformation

of mouse 3T3 cells. The vector also contained the mouse DHFR gene, such that culture of transformants in medium with increasing concentrations of methotrexate resulted in amplification of inserted DNA. The level of antisense transcripts is strongly increased by culture of transformants in medium with 65 μM Zn^{2+} . And, after four days of culture with Zn^{2+} , the β -polymerase level had decreased by 4 to 5-fold. Interestingly, we found that repair of DNA damage after nitrogen mustard exposure of the Zn^{2+} induced antisense cells was blocked. The results indicate that sudden down regulation of β -pol expression alters (blocks) cell DNA repair in mouse 3T3 cells.

Publications:

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05231-17 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
D. Guerini	Visiting Fellow	LB	NCI
M.P. Strub	Visiting Fellow	LB	NCI
P. Stemmer	IRTA Fellow	LB	NCI
F. Suprynowicz	NRC Fellow	LB	NCI
H. Ren	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any)

Dr. A. Bax and M. Ikura, NIDDK; Dr. Craig Montell, Johns Hopkins University;
 Dr. O. Wesley McBride, Laboratory of Biochemistry, NCI; Eva Mezey, NIMH; K.
 Beckingham, Rice University, Houston, Texas

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Protein Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

6.50

PROFESSIONAL:

6.50

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

The mechanism of stimulus-response coupling mediated by Ca^{2+} was studied at three levels: the modulation of the Ca^{2+} signal by calmodulin, the regulation of the Ca^{2+} and calmodulin-stimulated protein phosphatase, calcineurin, and the role of protein dephosphorylation in the control of mitosis. 1) We reported earlier that occupancy of two of the four Ca^{2+} sites of calmodulin destabilizes the hinge in the central helix of calmodulin between residues 74 to 77. Formation of a complex of calmodulin with a calmodulin-binding peptide changes the same residues from an α -helical to an extended conformation. Thus, Ca^{2+} -induced changes in the central helix may facilitate the interaction of calmodulin with its targets. 2) Calcineurin was shown to contain high affinity Ca^{2+} sites saturated at resting Ca^{2+} levels and low affinity sites responsible for the small, calcineurin B-mediated, activation of the phosphatase activity at micromolar Ca^{2+} concentrations. At physiological Ca^{2+} levels in stimulated cells (0.5-1 μM), the Ca^{2+} -dependence of the large cooperative activation of calcineurin is dependent on calmodulin concentration. 3) The inactivation of the mitosis-specific cdc2 kinase accompanying the metaphase to telophase transition has been investigated by Dr. Suprynowicz. It is ATP-dependent, okadaic acid sensitive and mediated by at least two factors, one of them expressed specifically at telophase. These results suggest that the concerted action of a protein kinase and a phosphatase is responsible for switching off cell division during mitosis.

Project Description

Objectives:

To study the functional roles of protein subunits and protein-protein interactions. The system under investigation is the Ca^{2+} -dependent regulation of enzymes mediated by calmodulin. Emphasis is on the mechanism of the regulation of 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 and Calcineurin with Calcium

In order to understand the mechanism of the Ca^{2+} -dependent activation of calmodulin-regulated enzymes, we have studied the Ca^{2+} -dependent conformational changes of calmodulin that allow binding to its targets. The secondary structure of Ca^{2+} -saturated calmodulin in solution determined by multi-dimensional NMR spectroscopy by Drs. Bax and Ikura revealed a considerable flexibility between Asp-78 and Ser-81 in the helix connecting the two halves of calmodulin. Complexing calmodulin with a peptide corresponding to the calmodulin-binding domain of myosin light chain kinase resulted in a change from α -helical to an extended conformation of residues 75 to 77. Occupancy of only two of the four Ca^{2+} sites of calmodulin also affects the conformation of residues 74 to 77 suggesting that this Ca^{2+} -induced destabilization of the central helix of calmodulin may facilitate the interaction of calmodulin with its targets (J. Mackall and M. Krinks).

The effect of calcineurin on the Ca^{2+} -binding to calmodulin was studied by Paul Stemmer. As reported last year, the affinity of calmodulin for Ca^{2+} is increased ten-fold in the presence of the calmodulin-binding domain of calcineurin. A k_d of $5 \times 10^{-7} \text{M}$ for Ca^{2+} -binding to the calmodulin/peptide complex is consistent with the Ca^{2+} -dependent, calmodulin-mediated, activation of calcineurin, which occurs between 10^{-7} and 10^{-6}M Ca^{2+} , within the physiological range of Ca^{2+} concentration in stimulated cells. The activation of calcineurin is dependent upon calmodulin concentration and is highly cooperative (Hill coefficient = 3) suggesting that more than two Ca^{2+} sites are involved in the activation process. The identity of the sites on calmodulin involved in this activation is being determined in collaboration with Dr. Beckingham using four calmodulin mutants which have lost the ability to bind Ca^{2+} at each of the four Ca^{2+} sites. Calcineurin is also under Ca^{2+} control mediated by its regulatory subunit, calcineurin B. Calcineurin contains two classes of Ca^{2+} sites. The high affinity sites, with a k_d of 10^{-10}M , are saturated at resting Ca^{2+} levels. The low affinity sites appear to be responsible for the small, calmodulin-independent activation of calcineurin by micromolar Ca^{2+} .

A proteolyzed derivative of calcineurin A which has preserved the ability to interact with calcineurin B but lost its calmodulin-binding and autoinhibitory domains cannot only be stimulated to its maximum activity in the absence of

calmodulin but its affinity for Ca^{2+} is increased ten-fold. It requires less than 10^{-7}M Ca^{2+} for activation. Thus, the regulatory domain of calcineurin A is not only involved in the calmodulin regulation but also exerts a negative control on the calmodulin-independent activation of the enzyme. The identification of the calcineurin A and calcineurin B interaction sites presently under way will enable us to use site-directed mutagenesis to dissect the roles of the two Ca^{2+} regulatory proteins.

B. Interaction of Calmodulin with Target Proteins

This project deals with the identification of the role of calcineurin in the regulation of cellular processes by protein dephosphorylation. The wide distribution and structural conservation of calcineurin in eukaryotes have been established by the cloning and sequence determination of the two subunits of calcineurin from human brain and Drosophila melanogaster by Danilo Guerini. In both organisms, the Ca^{2+} -regulatory subunit, calcineurin B, is the product of a single gene. The human and Drosophila proteins are 88% identical. In collaboration with Dr. McBride, we have shown that the two isoforms of human calcineurin A are the products of genes located on chromosomes 4 and 10. Dr. Mezey has recently shown that these two genes are differentially expressed in different regions of the brain and other tissues. Likewise, two different forms of calcineurin A are expressed in Drosophila. The human and Drosophila proteins differ in their amino and carboxyl termini but retain a conserved catalytic domain homologous to the catalytic subunits of protein phosphatase-1 and -2A as well as a regulatory domain specific to calcineurin. The intron-exon structure of the Drosophila calcineurin A1 gene correlates well with the structural motifs constituting the calmodulin-binding and autoinhibitory domains, the divergent amino termini and two almost perfectly conserved sequences whose functional role remains to be identified. The catalytic center is encoded by six exons. All intron-exon junctions so far identified in the human genes are also present in the Drosophila gene.

C. Role of Protein Phosphatases in Mitosis

There is a growing body of evidence that a major mechanism to orchestrate cell division in eukaryotes involves a complex interplay of protein kinases and phosphatases. Dr. Suprynowicz has obtained preliminary evidence for the involvement of these two classes of enzymes in the metaphase to telophase transition. During the past year he has studied the mechanism of inactivation of the mitotic-specific cdc2 kinase during telophase. He has demonstrated the presence in cytosolic extracts of at least two factors required for the inactivation of cdc2 kinase. One of these factors is telophase-specific whereas the other is active throughout all stages of mitosis. The ATP-dependence and okadaic acid-sensitivity of the inactivation of cdc2 kinase suggests the involvement of a kinase and a phosphatase in the turning off of the mitotic specific cdc2 kinase. The purification and characterization of the two factors will help us to understand how cell division is switched off during mitosis.

Publications:

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

PROJECT NUMBER

Z01 CB 05244-14 LB

PERIOD COVERED
 October 1, 1990 to September 30, 1991

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
L. Mizrokhi	IRTA	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; J.E. Celis, Institute of Medical Biochemistry and Bioregulation Research Center, Aarhus University; Alexander Mazo, 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:
5.50	5.25	0.25

CHECK APPROPRIATE BOX(ES)

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

(a1) Minors B

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
 Models for the transposition of the LINE-1 human retrotransposon (L1Hs) require transcription, translation, and reverse transcription prior to insertion into new genomic locations. The absence of long terminal repeats from these elements suggested that all these processes might be mechanistically distinct from the analogous processes operative in the retrotransposition of elements that, like retroviruses, have long terminal repeats. Our recent experiments reveal just such unusual mechanisms. Thus, transcription of L1Hs, which is efficient in only a few of the human cell types tested including teratocarcinoma, starts at residue 1 of the 6 kbp element and is regulated by sequences spread over 660 bp downstream from the start site within a 900 bp 5' untranslated region. Multiple sequences extending throughout the first (essential) 100 bp contribute to regulation. Another region (residues 385 to 525) stimulates transcription in an orientation-independent and cell type-dependent manner, suggesting that it contains a cell-specific enhancer. The polypeptide (p40) produced by ORF1 *in vitro* and in cells is consistent with translation initiation at an AUG almost 900 residues downstream of the start of the mRNA. It appears that ribosome binding may occur at internal sequences. Consistent with this is the fact that *in vitro* translation appears to be cap-independent and yields identical polypeptide products from ORF2 regardless of whether ORF1 is present or translatable; no evidence for an ORF1/ORF2 fusion product is observed.

The electrophoretic mobility (under denaturing conditions) of the p40 produced by a putatively active L1Hs, L1.2 (upon transient transfection), is the same as that of the endogenous protein. Yet, one cDNA representing L1Hs mRNA yields a p40 protein of somewhat lower mobility although the size (but not the amino acid sequence) of the ORF1 is identical in the cDNA and L1.2. This could reflect different post-translational modifications or structural changes; p40 appears to be phosphorylated. Evidence indicating the horizontal transmission of the LINE-like jockey element of *D. melanogaster* has been obtained.

Project Description

Objectives:

We have continued to work on the human transposable element, LINE-1 (L1Hs). L1Hs, and similar retrotransposons in a wide variety of eukaryotes, are characterized by an absence of long terminal repeats, a polyA stretch at the 3' end of the strand that carries long open reading frames, and coding sequences that predict a reverse transcriptase. Our experiments concentrate primarily on the transcription and translation of L1Hs, events that are presumed to be required for transposition. Previously, we demonstrated that the specific transcription of L1Hs in teratocarcinoma cells depends on internal transcriptional regulatory signals within the 900 bp 5' UTR. We now aim to characterize the cis-acting DNA motifs and the transcription factors that regulate L1Hs transcription. These regulatory elements appear to be active in only a limited number of cell types. Moreover, they are unusual in that they function downstream of the start of transcription.

Consideration of the structure of L1Hs mRNA suggests that translation also depends on unusual mechanisms. Thus, we would like to understand the mechanism by which ORF1 translation initiates 900 bp downstream of the start of the RNA. We would also like to know whether translation of ORF2 depends on reinitiation or some type of suppression of the stop codons separating ORF1 from ORF2.

In addition, we are interested in studying the distribution and evolutionary history of the elements: for these studies, the Drosophila melanogaster LINE-1-like element called jockey is being investigated.

Major Findings:

A. L1Hs Transcription

This work concerns two aspects of the regulation of L1Hs transcription: 1) cell-type specificity and 2) nature of the regulatory signals. The approach is to measure the expression of reporter genes surrounded by the 5' and 3' untranslated regions (or modified derivatives thereof) of an L1Hs element, in transient transfection experiments using cells in culture.

This year, we demonstrated that L1Hs transcription is relatively high in mouse teratocarcinoma cells as well as human ones. Thus, in NIH-3T3 and P19 cells, the expression of the reporter gene was 1 and 10 percent of the expression driven by the SV40 early promoter, respectively. Although mouse and human L1 5' UTRs share little, if any, DNA sequence, it appears that components of mouse teratocarcinoma cells may recognize the cis-acting regulatory sequences in the L1Hs 5' UTR.

Last year we reported that the first 100 bp of L1Hs 5' UTR are essential for transcription. This region has now been investigated in some detail. Deletions starting at -32 (transcriptional start site is +1) and extending to +11, +18, +32, and so forth, indicate a progressive decrease in transcription as sequences beyond +11 are deleted. One region, residues 78 through 93, was of special interest because it contains two overlapping

potential OCTA binding sites and an OCTA binding protein, OCT3, has been shown (by others) to function as a transcriptional regulatory factor specifically in teratocarcinoma cells. However, although deletion of residues 71 to 99 reduced gene expression 20 fold, replacement of residues 78-85 or of 88-93 or of 78-93 with sequences known not to bind OCT3 diminished expression in only minor ways. Thus, modulation of expression by this region of the 5' UTR does not appear to involve the OCTA binding sequence itself. This conclusion is consistent with the failure to observe enhanced expression in experiments in which the L1Hs plasmid construct was cotransfected into HeLa cells (generally poor expression) along with a plasmid that expresses the murine OCT3 binding protein (kindly provided by Dr. Louis Staudt, NIH).

Experiments designed to identify cell-type specific enhancers in the L1Hs 5' UTR have been carried out. These studies suggest that the segment between +385 and +525 possesses orientation-independent enhancer activity that is strong in teratocarcinoma cells (NIERA2D1) and weak in other cell types.

B. Translation of L1Hs Coding Regions

As reported last year, the polypeptide product (p40) of the L1Hs ORF1 (the 5' most of the two ORFs) is readily detectable in certain human cells in culture using the specific antibodies we had prepared against protein synthesized in *E. coli*. Last year we also described plasmid constructs that can be used to study in vitro transcription/translation and for transfection protocols. We have now initiated studies on the properties of p40 and on the translation of ORF2 (using antibodies prepared as described for ORF1).

Properties of p40. In collaboration with J.E. Celis, p40 was shown (by 2-dimensional gel electrophoresis) to be the size predicted from the DNA sequence, 40.3 kDa, and to consist of a mixture of molecules, some with a pI of 10.3 (as predicted from amino acid sequence) and others with a range of acidic pI's. Consistent with this, a major portion of the endogenous p40 in 2101EP teratocarcinoma cell cytoplasm appears to be phosphorylated at multiple sites, as demonstrated by SDS PAGE electrophoresis before and after treatment with potato acid phosphatase. Analysis of the predicted p40 structure revealed the presence of a "leucine zipper".

A431 (squamous cell carcinoma) cells contain an amount of p40 similar to that found in teratocarcinoma cells; multiple bands of immunoreactive material are frequently observed. p40 from 2102EP cells separates into two peaks on Sephacryl-S400 column chromatography. The second peak, which elutes in the vicinity of the 44 kDa marker, is the only peak obtained if the cytoplasmic extracts are prepared in the presence of Triton-X100.

Kazazian and coworkers have tentatively identified one allele (L1.2B) of an L1Hs element located on human chromosome 22, L1.2, as an actively transposing unit. A cloned allele, L1.2A, which is identical to L1.2B in the 5' UTR (untranslated region), ORF1, and 3' UTR, has been used to study ORF1 expression. In contrast to the p40 produced by a cloned L1Hs cDNA (reported last year), the ORF1 of L1.2A yields a p40 (upon transient transfection of teratocarcinoma cells) with the same mobility as the

endogenous p40. This result supports the hypothesis that L1.2B is an active transposable unit.

Translation of ORF1 and ORF2. The L1Hs ORF2 is separated from ORF1 by several in-frame stop codons and about 30 bp (the interORF region); ORF2 predicts a polypeptide of 140 kDa and appears to encode a reverse transcriptase. In vitro transcription/translation studies using L1.2A sequences and a reticulocyte lysate system indicate that 1) ORF2 translation is independent of ORF1 translation; 2) translation of ORF1 and ORF2 is not dependent on the presence of a 5' cap; 3) translation initiates internally and at more than one site on L1.2A RNA.

C. Horizontal Transmission of LINE Elements in Drosophila

The striking similarity in structures of LINE-like elements among the very distant species (e.g. mammals, Drosophila and trypanosoma indicates an evolutionary relation. The relatively rapid rate of divergence intrinsic to reverse transcriptase-dependent elements leaves horizontal transmission as a likely explanation for such a similarity. Last year we reported the unusual distribution of the LINE-like jockey element; although two distantly related species D. melanogaster and D. funebris contain jockey elements, species more closely related to D. melanogaster do not. Now we have demonstrated that species sibling to D. funebris also lack jockey. This indicates that horizontal transmission must have occurred.

Publications:

Mizrokhi LJ, Mazo AM. Evidence for horizontal transmission of the mobile element jockey between distant Drosophila species, Proc Natl Acad Sci USA 1990;87:9216-20.

Leibold DM, Swergold GD, Singer MF, Thayer RE, Dombroski BA, Fanning TG. Translation of LINE-1 DNA elements in vitro and in human cells, Proc Natl Acad Sci USA 1990;87:6990-4.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05258-12 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
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M. Shirakata	Visiting Fellow	LB	NCI
R. Horowitz	Staff Fellow	LBP	NIAMS
S. Nakamura	Visiting Scientist	LB	NCI
C. Vinson	Staff Fellow	LB	NCI

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

7.50

PROFESSIONAL:

7

OTHER:

0.50

CHECK APPROPRIATE BOX(ES)

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

B

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

We have isolated and characterized a number of muscle-specific as well as generally expressed cellular genes that are differentially regulated during muscle formation (myogenesis) in vitro. These include the muscle-specific genes for alpha cardiac actin, alpha skeletal actin, the myosin light chains 1f/3f, and the ubiquitously expressed beta actin gene. Through deletion and transfection analyses, we have been able to define minimal cis-acting regions responsible for developmental regulation of these genes in a muscle cell background. In addition to a variety of generic transcription factors, the muscle-specific genes require the presence of additional muscle-specific factors, the MyoD family of proteins, which are required not only for specific muscle transcription but also for cellular determination of the muscle phenotype. We have since focused out studies on the mechanism of action of two of the four MyoD-like genes isolated in the vertebrates: the avian MyoD, called CMD1, and the avian myogenin, called Cmgm. We have also isolated the single homologue to the vertebrate genes from Drosophila, called Dmyd (Drosophila myogenic determination factor), in order to study the function of these genes genetically and biochemically. Such an approach should enable us to study the upstream and downstream genes in the myogenic determination pathway and to look for correlates in the vertebrate system.

Project Description

Objectives:

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

Major Findings:

A. Characterization of Two Avian Myogenic Factors

We have isolated the cDNA encoding a myogenic factor expressed in embryonic chick breast muscle by virtue of its weak hybridization to the mouse MyoD1 clone. Nucleotide sequence analysis and amino acid comparison define this clone, called CMD1, as encoding a protein similar to mouse MyoD1. CMD1 encodes a polypeptide smaller than MyoD1, 298 versus 318 amino acids, respectively, and is 80% concordant by amino acid sequence overall. The basic and helix-loop-helix (BHLH) domains required for myogenic conversion of 10T1/2 fibroblasts and muscle promoter activation are completely conserved in the avian and mouse MyoD proteins. In fact, the mouse and avian proteins are virtually identical in all assays used to define protein function (conversion, muscle promoter activation, auto activation, MCK enhancer binding with E12). An interesting observation with CMD1 expression plasmids in 10T1/2 cells suggests the efficiency of conversion depends upon the level of expression of CMD1: high levels of expression convert single cells to muscle with concomitant withdrawal from the cell cycle and loss of the cell from the putative myoblast population, whereas low levels of CMD1 expression convert 10T1/2 cells to muscle while maintaining a dividing myoblast population. This suggests that MyoD levels (CMD1) may play a role not only in maintaining the muscle phenotype but also in the timing of withdrawal of the myoblast from the cell cycle and its subsequent differentiation. Cmg1, the avian homologue to myogenin (see below), does not do this so the effect appears to be CMD1 specific. We would like to look at this regulation in more detail. Growth of muscle cells in the thymidine analogue bromodeoxyuridine (BUdR) reversibly inhibits myogenesis and the expression of the myogenic factor CMD1. Constitutive expression of CMD1 in BUdR treated muscle cells will activate muscle-specific promoters and trigger myogenesis, suggesting that the inhibitory effect of BUdR is, in part, the inactivation of CMD1 expression. This is under investigation.

Using the human myogenin clone, Myf4, we were able to isolate the avian homologue, Cmg1, from the same cDNA library used to isolate CMD1. The clone is 1206 nucleotides long, contains a poly A addition site and a 29 nucleotide leader sequence. It encodes a polypeptide of 226 amino acids and has a conserved BHLH domain common to all the myogenic factors. Within the 61 amino acids of this domain, there are 12 amino acid changes compared to CMD1 and 2 or 3 changes compared to human and mouse myogenin, respectively. Cmg1 will convert 10T1/2 fibroblasts to muscle, activate muscle-specific promoters, and bind to the MCK enhancer in a complex with E12 similar to CMD1. However, Cmg1 levels of expression do not appear to affect the conversion efficiency or the ability to establish myogenic lines as is the case for CMD1.

The CMD1 gene has also been isolated and the promoter is under investigation since CMD1 expression is autoregulated and inhibited by BUdR. At present we know the promoter is muscle specific and inhibited in BUdR treated cells with deletions down to -169 (transcription starts at plus 1) but we have not been able to show autoactivation in CMD1 cotransfections with a reporter construct containing either 1 kb or 8 kb of promoter fragment. We are trying to see if the cotransfection of additional muscle factors with CMD1 will result in activation of either promoter construct. We do know that the entire 9 kb gene for CMD1 with 1 kb of promoter will convert 10T1/2 cells to muscle so the postulated upstream gene, Myd1, does not appear to be necessary. In addition, the 1 kb promoter fragment joined to the CMD1 cDNA will also convert cells. The CMD1 cDNA without a promoter will not convert cells, suggesting that the conversion is promoter specific and not due to leaky nonspecific transcription from the plasmid.

B. Baculovirus Expression of Myogenic Factors

In an effort to study the biochemical properties of CMD1, we have expressed this protein in the Baculovirus system since it is a eukaryotic expression system that gives reasonable yields of appropriately modified protein that more closely resembles the protein expressed in muscle cells. The protein has been purified to homogeneity by immunoaffinity chromatography and has been shown to be a phosphoprotein as reported for the authentic muscle protein. When this protein is injected into muscle cells it is nuclear in location. We are testing its ability to convert 10T1/2 cells, for this appears to be dosage dependent. The BaculoCMD1 will bind with E12 made in retic lysates and bind specifically the E box motif of the MCK enhancer, as determined by band shift with normal and mutant oligos as well as footprint assays. The most interesting result suggests the phosphorylation of CMD1 has a tremendous influence on its ability to bind to the MCK enhancer in a complex with E12: the dephosphorylated protein does not bind well but it can be rephosphorylated with cell extracts and binding is restored. We are in the process of mapping the phosphoamino acids so we can mutate these sites and study the function of the mutated CMD1 in conversion and activation assays.

C. NMR Analysis of CMD1

In order to pursue structural studies we have succeeded in producing the BHLH domain of CMD1, the HLH domain of E12, and the HLH domain of chicken E47 in the T7 system of Studier. We hope to carry out preliminary studies with the Ad Bax group on the nature of the CMD1 BHLH region and the CMD1-E47 complex with and without the minimum DNA fragment containing an E-box binding domain.

D. Isolation of the Avian Homologues to E47/E2A and ID

Using the mammalian sequences for E12 and ID we have designed PCR primers to generate probes that were used to screen avian genomic and cDNA libraries for the homologues to these sequences. We have isolated a 1500 nucleotide clone that is the homologue to E47/E2A in chicken. Remarkably, this mRNA appears to be highly expressed in muscle as compared to brain or liver. This could be a good candidate for the partner to CMD1 in muscle. Similar screenings have yielded potential candidates for ID and these are under investigation.

E. The isolation of the Drosophila Homologue of the Vertebrate Myogenic Determination Gene, Dmyd

We have isolated a cDNA clone, called Dmyd for Drosophila myogenic determination gene, that encodes a protein with the structural and functional characteristics similar to the members of the vertebrate MyoD family. Dmyd 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 indicate Dmyd is not a member of a multigene family similar to MyoD in 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 8 kb promoter fragment from the gene, which contains the first 55 amino acids of the Dmyd protein, joined to lacZ to follow myogenic precursor cells into muscle fibers using antibodies to beta galactosidase and Dmyd. Unlike the myogenic factors in vertebrate muscle cells, Dmyd appears to be expressed at a much lower level in differentiated Drosophila muscles so it cannot be followed continuously as a muscle marker. This is reflected in the loss of expression of Dmyd RNA in 12-24 hour embryos, a major period of early myogenesis, as well as in the undetectable level of nuclear antigen in primary cultures of embryonic and adult Drosophila muscle. We are currently in the process of trying to isolate a mutant for the Dmyd gene by P element insertion mutagenesis. This will be used in conjunction with our studies on the upstream and downstream genes involved in Drosophila myogenesis. Information gained here can be used to study vertebrate myogenesis in a manner similar to the homeotic gene studies in vertebrates (Dr. Paterson was on sabbatical in Basel, Switzerland in Dr. Walter Gehring's lab to study the Drosophila Dmyd gene and to develop the system for further studies).

F. The Function of Dmyd in Vertebrate Myogenesis

Our preliminary studies with Dmyd in 10T1/2 fibroblasts indicated that although the BHLH was essentially conserved, Dmyd did not convert these cells to muscle even though it was well expressed in these cells and a good nuclear antigen. Furthermore, it weakly activated a vertebrate muscle-specific promoter in cotransfections and bound poorly to the MCK enhancer in a gel shift with E12. In order to analyze this further, we have started to construct chimeric proteins between Dmyd and CMD1 to define the activation and conversion domains in CMD1 more precisely. Preliminary results suggest the activation domain is located in the acidic amino terminal region of CMD1, that the basic domains are equivalent, that the helix regions are different, and that the BHLH of CMD1, even though it is a weak activator, will convert the cells. This may tie into the phosphorylation studies since regions outside the BHLH are so different in Dmyd and CMD1.

G. The Myosin Light Chain 1f/3f Gene

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

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

H. Studies on the Yeast Myosin Gene

Recent studies with myosin heavy chain (MHC) mutants of *Dictyostelium* and the yeast *Saccharomyces cerevisiae*, suggest the MHC gene is not essential for cell survival under laboratory growth conditions. However, cells lacking a normal MHC demonstrate substantial alterations in the normal cell division pattern. In this study, we report that a disruption mutant in the rod portion of the yeast MHC gene produces an abnormal primary septum and cell wall organization at the mother-bud neck in a high proportion of dividing cells. It is suggested that this phenotype is the cause of the cell division defect and the osmotic sensitivity of yeast MHC mutants. In the absence of a normal MHC polypeptide, cells lose their cell type specific budding pattern with a concomitant loss of normal growth polarity. It is concluded that an intact MHC gene is required to maintain the cell type specific budding pattern and the correct localization and deposition of septum and cell wall components during cell growth and division.

Publications:

Peterson CA, Gordon H, Hall ZW, Paterson BM, Blau HM. Negative control of the helix-loop-helix family of myogenic regulators in the NFB mutant, *Cell* 1990;62:493-502.

Rodriguez JR, Paterson BM. Yeast myosin heavy chain mutant: maintenance of the cell type specific budding pattern and the normal deposition of chitin and cell wall components requires an intact myosin heavy chain gene, *Cell Motility and Cytoskeleton* 1990;17:301-8.

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.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05262-11 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Eukaryotic Gene Regulation and Function: The Metallothionein System

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PROFESSIONAL:

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OTHER:

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CHECK APPROPRIATE BOX(ES)

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

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

Transcriptional control by metal ions provides a useful paradigm to study eukaryotic gene regulation. When yeast cells are exposed to Cu, they repress transcription of the gene for cell surface reductase and activate transcription of the gene for metallothionein. Reductase catalyzes the conversion of Cu(II) to Cu(I), a new reaction discovered in this lab, whereas metallothionein chelates Cu and is involved in detoxification. The major cell surface reductase is encoded by FRE1, which was previously identified as an Fe reductase, while an alternative form is revealed in upc31 mutant strains. The major regulator of metallothionein gene transcription is ACE1, which contains a novel "copper fist" structure based on a polynuclear Cu(I)-S cluster. A second regulatory pathway is controlled by a heat shock transcription factor. Three proteins that regulate metallothionein gene transcription in mammals have been identified and are being characterized at the molecular level.

Projection DescriptionObjectives:

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

Major Findings:A. Copper Reduction: Down Regulation of FRE1

1. LIVING CELLS CAN REDUCE CU(II) BY TWO PATHWAYS. The overwhelming form of copper in the environment is Cu(II) whereas the predominant form inside of cells is Cu(I). It had previously been assumed that the necessary reduction step was an intracellular, nonenzymatic, constitutive process. To test the alternative notion of enzymatic reduction, intact yeast cells were incubated with CuSO₄, a Cu(II) salt, and bathocuproine disulfonate, a redox-sensitive chelator that generates a colored complex in the presence of Cu(I). The results showed that yeast cells contain an enzymatic reductase activity that is directed to the outside of the cell. Further experiments demonstrated that this cell surface reductase is encoded by the FRE1 locus, which was previously identified by R. Klausner and coworkers as a Fe(III) and K₃Fe(CN)₆ reductase involved in iron utilization. The sequence of the FRE1 gene, determined by Klausner et al., predicts a cell surface protein with homology to other oxioreductases.

In an attempt to identify additional pathways for Cu(II) reduction, a collection of mutants with defects in copper metabolism were assayed for external reductase activity. The upc31 mutant, which was isolated on the basis of increased basal metallothionein gene (CUP1) transcription, was found to overproduce cell surface reductase. Experiments with frel-Δ upc31 double mutants demonstrate that this pathway is independent of reduction by the FRE1 protein. The upc31 gene has been cloned and is currently being analyzed.

2. Cu(II) REDUCTION IS BIOLOGICALLY RELEVANT. The biological role of Cu(II) reduction was determined by comparing cells in which cell surface reductase activity is completely lacking (frel-Δ), present in normal quantities (wt), or overproduced (upc31). The results showed that reductase-minus cells are deficient for the utilization of low concentrations of copper and are resistant to high levels of copper. Conversely, reductase overproducers transport increased quantities of copper into the cell and are copper-sensitive. Therefore the reduction of external copper is critical for both the growth stimulating and inhibitory effects of this metal.

3. CU(II) REDUCTION IS REGULATED. To determine whether the reduction of external Cu(II) is a regulated or constitutive process, cells were grown in the presence of various copper concentrations and tested for reductase activity, FRE1 messenger RNA, and the expression of a FRE1::lacZ fusion gene. The results showed that copper ions strongly repress expression of FRE1 at the transcriptional level. This form of regulation is exquisitely sensitive to low concentrations of Cu(II) and is independent of the ACE1/CUP1 pathway. Attempts to isolate the trans-acting proteins and cis-acting promoter sequences responsible for copper down-regulation are under way.

B. Copper Chelation: Up Regulation of CUP1

1. BIOPHYSICAL CHARACTERIZATION OF ACE1. When yeast cells are exposed to a high concentration of Cu, they respond by increasing the transcription 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 proposed that the amino-terminal domain of ACE1 forms a polynuclear Cu(I)-S cluster, or "copper fist", which organizes the protein into a specific DNA binding factor. This proposed structure is fundamentally different than that found in any other DNA-binding protein.

In order to test this model, the amino-terminal domain of ACE1 was overproduced in bacteria, purified to homogeneity, and characterized by biophysical techniques including reconstitution, ultraviolet spectroscopy, luminescence spectroscopy, extended X-ray absorption fine structure analysis, and circular dichroism. The results, which were obtained in close collaboration with the laboratory of D. Winge, showed that ACE1 contains a polynuclear Cu(I)-cysteiny l thiolate cluster that is remarkably similar to the structural core of metallothionein itself. The cluster consists of 6-7 Cu(I) ions coordinated to cysteiny l thiolates in a trigonal geometry distorted from planarity. This Cu cluster organizes and stabilizes the conformation of the amino-terminal domain for specific DNA binding. Now that reconstituted, homogeneous samples of Cu-ACE1 are available, it may be possible to determine a three-dimensional structure by X-ray crystallography or NMR.

2. ROLE OF COACTIVATORS IN TRANSCRIPTIONAL REGULATION BY ACE1. Once Cu-ACE1 has bound to the CUP1 gene promoter through its amino-terminal domain, it activates transcription by a reaction that requires the acidic carboxy-terminal domain. Using an in vitro transcription system in which ACE1 is active, we analyzed the sequence and factor requirements for this reaction. We showed that both basal and induced CUP1 gene transcription require a functional TATA sequence and the TATA-binding factor TFIID. While crude TFIID preparations from mammalian cells responded to stimulation by ACE1, purified proteins expressed from the cloned gene did not. Further experiments demonstrated that additional factors, which we term "coactivators", are necessary for induction. Similar results were simultaneously reported by several other laboratories, suggesting that coactivators play a role in the regulation of many eukaryotic genes.

C. Activation of CUP1 Gene Transcription by Heat Shock Factor

In an effort to detect new stimuli and factors that regulate metallothionein gene transcription, we isolated a Cu-resistant suppressor mutant of an ACE1 deletion strain. Even in the absence of metals, the suppressor mutant exhibited high basal levels of metallothionein gene transcription that required upstream promoter sequences. The suppressor gene was cloned and shown to correspond to yeast heat shock transcription factor with a single amino acid substitution in the DNA-binding domain. The mutant heat shock factor bound strongly to metallothionein gene upstream promoter sequences whereas wild type heat shock factor interacted weakly with the same region. Heat treatment led to a slight but reproducible induction of metallothionein gene expression in both

wild-type and suppressor strains, and Cd induced transcription in the mutant strain. These studies provide the first evidence for multiple pathways of metallothionein gene transcriptional regulation in yeast.

D. Regulation of Mammalian Metallothionein Gene Transcription

1. MBF1, A CONSTITUTIVE MRE-BINDING PROTEIN. Now that the basic mechanism for metallothionein gene regulation in yeast has been worked out, interest in mammalian systems has revived in the laboratory. We showed previously that the mouse and human nuclear factor MBF1 binds to certain metallothionein gene metal regulatory elements (MREs) in a sequence-specific fashion independent of metal ions. In collaboration with J. Imbert, a former postdoc now working in France, human MBF1 has been purified to homogeneity and partial tryptic peptide sequences have been obtained. These are being used to clone the MBF1 gene and determine its relevance to basal and induced metallothionein gene transcription.

2. ZAP1, A ZINC-REGULATED FACTOR. In agreement with reports from other laboratories, we have found that mouse and human cells contain a factor that binds to MRE sequences in a zinc-dependent fashion. The chelation and reconstitution properties of this factor, ZAP1, suggest that zinc plays a regulatory rather than constitutive structural role. Efforts to purify ZAP1, which is present in low concentrations, are under way.

3. A YEAST GENETIC SCREEN FOR MAMMALIAN REGULATORY FACTORS. A general scheme to select for sequence-specific transcription factors by genetic complementation in yeast has been devised and applied to the isolation of metallothionein gene regulatory factors. Yeast cells were simultaneously transformed with a selector construct, in which an MRE drives the expression of the galK gene, and an expression construct containing random mouse cDNA sequences in a yeast high copy vector. One cDNA, which activates expression of the MRE::galK fusion gene, but not various control constructs, has been isolated and is being sequenced. This method may be useful for isolating a variety of regulatory factors that are difficult to clone by standard screening techniques.

Publications:

Hu S, Furst P, Hamer D. The DNA and Cu binding functions of ACE1 are interdigitated within a single domain, *The New Biologist* 1990;2:544-55.

Kambadur R, Culotta V, Hamer D. Cloned yeast and mammalian transcription factor TFIID gene products support basal but not activated metallothionein gene transcription, *Proc Natl Acad Sci USA* 1990;87:9168-72.

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*, in press.

Yang W, Gahl W, Hamer DH. Role of heat shock transcription factor in yeast metallothionein gene expression, *Mol Cell Biol*, in press.

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

PROJECT NUMBER

Z01 CB 05263-10 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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J. L. Brown	Visiting Associate	LB	NCI
S. Rabindran	Staff Fellow	LB	NCI
G. Lavorgna	Visiting Associate	LB	NCI
J. Clos	Visiting Fellow	LB	NCI
P. Becker	Visiting Fellow	LB	NCI
J. Westwood	Guest Researcher	LB	NCI
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7.75

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

This group has continued its study of sequence-specific DNA-binding proteins in *Drosophila*, with focus on transcription factors regulating heat shock genes and the segmentation gene *fushi tarazu*, *ftz*. Three transcription factors: heat shock factor (HSF), FTZ-F1, and FTZ-F2 (now referred to as tramtrack), previously cloned in the laboratory have been expressed in *E. coli*, and the recombinant proteins have been utilized for biochemical studies and for the preparation of antibodies. The antibodies have been employed to determine the subcellular distribution of the transcription factors. In addition, a human heat shock transcription factor has been cloned by PCR using local amino acid identities between the *Drosophila* and yeast HSF protein sequences. The cloning of a human HSF paves the way for a detailed study of the induction of this important physiological response in mammalian cells.

Project DescriptionObjectives:I. Induction of Heat Shock Genes

A. Analysis of the Drosophila HSF Gene Intron-Exon Structure (J. Clos)

Using the previously cloned Drosophila HSF cDNA, we have isolated and sequenced 4.8 kbp of Drosophila genomic DNA encoding HSF. At least six introns split the sequences coding for the HSF mRNA. Interestingly, the DNA sequences coding for the DNA binding domain of HSF are derived from three exons in the genomic DNA. In particular, sequences coding for a dodecapeptide which is highly conserved between the HSFs of all species examined so far and which displays similarities to a putative helix-turn-helix motif of bacterial sigma factors are split by an intron. Low stringency Southern blot analysis indicates that the Drosophila genome contains only one gene coding for a HSF in contrast to mammalia and plants where two and more genes have been reported.

B. Expression and Functional Analysis of Drosophila HSF Protein (J. Clos)

Recombinant HSF expressed in E. coli bacteria shows an inherent activity both as DNA binding protein and as transcription factor in vitro. After denaturation by heat the protein refolds spontaneously into an active conformation. Native size determination of recombinant Drosophila HSF by non-denaturing gel electrophoresis and cross-linking studies suggest the active form to be a homo-hexamer of ca. 700 kDa. Further studies are in progress to determine the molecular weight of native HSF.

Deletion of the sequences implicated in self-association of the HSF complex, namely a highly hydrophobic stretch of amino acids with three potential leucine zipper motifs, reduces the affinity of HSF for its cognate DNA sequence 250-fold without impairing the specificity of binding. A fourth potential leucine zipper motif found near the C-terminus of the protein is speculated to be involved in the negative regulation of the HSF activity in the Drosophila cell. We are currently trying to express Drosophila HSF and deletion mutants thereof in cultured Drosophila cells to identify the domains involved in the regulatory events.

C. Cloning and Expression of a Human HSF Protein (S. Rabindran, G. Giorgi)

The gene encoding the human heat shock factor (HSF1) was cloned by using the polymerase chain reaction (PCR) with primers designed from the conserved amino acid residues between the Drosophila and yeast factors. The fragment produced by PCR was then used as a hybridization probe to screen a recombinant cDNA library from human lymphoid cells. The DNA sequence for human HSF reveals an open reading frame (ORF) of 529 amino acids and encodes a protein with four leucine zipper motifs. A variant of this clone was also isolated from the same library which encodes a protein of 489 amino acid residues. The two clones are identical except for a heterogeneity at the 3' end of the sequence. A comparison of the cDNA clone with a partial genomic clone indicates that the heterogeneity may be due to an alternative splicing mechanism.

Both ORFs were expressed in bacterial cells using the T7 expression system and were shown to function as DNA binding transcription factors in the absence of a heat shock. Similar results were obtained by expression of the protein in rabbit reticulocyte lysates. This is reminiscent of the *Drosophila* situation and suggests that *in vivo* the activity of the human HSF is under negative control. Experiments to introduce the cloned HSF into mammalian systems by transfection are now in progress. Preliminary deletion analyses of the protein implicate the C-terminal half of the protein as the transcriptional activation domain, while the N-terminal half is organized into a DNA binding domain and an oligomerization domain required for efficient binding. In these respects the human HSF appears to be similar to those isolated from *Drosophila* and yeast. Surprisingly, a second heat shock factor (HSF2) was isolated independently by a different group. This protein is related to but distinct from HSF1. The presence of two heat shock factors in humans suggests that the two factors may respond to different stress inducers.

Recombinant HSF1 has been purified from bacterial extracts and used to raise antibodies. Both polyclonal and monoclonal antibodies were obtained and are currently being characterized. These reagents appear to be specific for HSF1 and will be used in Western blots, immunoprecipitations and cell staining procedures in an effort to study the regulation of HSF and the stress response in living cells. The availability of these specific reagents and the cloning of the two HSF genes will pave the way for a detailed dissection of this important physiological response in mammalian cells.

D. Regulation of *Drosophila* HSF Activity (J.T. Westwood)

In unshocked cells *Drosophila* HSF is present in an inactive, non-DNA binding state. Upon heat shock or exposure to other forms of stress, the DNA binding and transcriptional activity of HSF is activated. We are investigating what happens to HSF upon hs and have raised polyclonal antisera and are currently making monoclonal antisera against *Drosophila* HSF to help study this process.

Fractionation of *Drosophila* Schneider line 2 (SL2) cells has revealed that HSF is found primarily in the cytosol in unshocked cells and in the nucleus in heat shocked cells suggesting that HSF is being translocated upon heat shock. We recently studied the location of HSF by immunocytochemistry and have found that HSF resides in the nucleus both before and after heat shock. This and other work has led us to conclude that HSF is a nuclear protein that apparently leaches out from the nucleus of unshocked cells during homogenization. The heat shocked form of HSF has a much higher affinity for DNA/chromatin and stays associated with the nucleus until it is extracted with high salt. We have also immunostained the giant polytene chromosomes of *Drosophila* third instar larvae and have found that in unshocked larvae, HSF is associated with the chromosomes with the staining being fairly uniform. In heat shocked larvae, the general staining is diminished and there is specific staining at more than a hundred different loci. The most intense staining has been generally found at the loci where the genes for the hs proteins are being transcribed, the heat shock puffs.

The region of HSF which is needed for its oligomerization has been identified. Deletion of this region results in a much lower affinity of HSF for the heat shock element (HSE) and this and other findings have suggested that

oligomerization of HSF is crucial for its binding to the HSE. We have examined the native size of the non-DNA binding and DNA binding forms of HSF by pore exclusion limit electrophoresis followed by Western blot analysis and have found that in unshocked extracts, HSF migrates as an approximately 220 kDa complex while in in vitro shocked extracts, the amount of HSF in the 220 kDa complex diminishes and a new complex of approximately 690 kDa appears. Gel-shift experiments with the same extracts on the same gels reveals that only the (in vitro) heat shocked extract binds to HSE and migrates as an approximately 690 kDa complex. Since the monomer size of HSF is 110 kDa (as determined by denaturing electrophoresis), the simplest interpretation of the above experiments is that the inactive form of HSF is a homodimer while the active form is a homohexamer. Other interpretations of these data are possible. For example if HSF is very asymmetrical, the inactive form could be a monomer and the active form a trimer. The inactive form could also be a heterodimer. Further experiments are being conducted to confirm the native size and composition of the inactive and active forms of HSF.

E. Chromatin Structure and Transcriptional Regulation of the hsp70 Gene (P. Becker)

We have used the nucleosome assembly system from Xenopus laevis oocytes to study the activity of the hsp70 promoter when assembled into chromatin in vitro. Increasing nucleosome density on the template DNA resulted in efficient inhibition of transcription when the plasmid was transcribed either in extracts from nonshocked or heat shocked Drosophila embryos. Incubation of the template with bacterially expressed yeast TFIID prior to nucleosome assembly did not lead to significant relief of inhibition when the subsequent transcription was performed with a nonshock extract. However, TFIID binding potentiated the hsp70 promoter to be responsive to later activation by heat shock factor. Order of addition and titration experiments confirmed that for efficient transcription of a heat shock promoter in chromatin, two basic requirements have to be met: TFIID has to bind to the template prior to nucleosome assembly (potentiation) and heat shock factor has to overcome the inhibition by nucleosomes in the later activation step. Heat shock factor is thus the first transcription factor with the demonstrated ability to activate transcription from a chromatin template.

To reconstruct the chromatin structure of Drosophila genes in vitro in a homologous system, we have developed a cell-free system for nucleosome assembly from the cytoplasm of preblastoderm Drosophila embryos. When double-stranded plasmid DNA is incubated in the extract under the appropriate conditions, long arrays of regularly spaced nucleosomes are formed with a repeat length of 180 bp. The nucleosomes are composed of the four core histones but lack histone H1. When purified H1 is added to the extract, it is incorporated into the chromatin resulting in an increased repeat length of about 195 bp. When single-stranded circular DNA is incubated in the extract, it is converted into closed double-stranded DNA in a reaction that is reminiscent of lagging-strand replication and that is accompanied by rapid nucleosome formation.

We are currently using the Drosophila nucleosome assembly extract as well as salt dialysis reconstitution of nucleosomes from purified histones to study the influence of nucleosomes on transcription factor/DNA interactions and, conversely, the possible effect of factor binding on the positioning of nucleosomes in vitro.

II. Regulation of the Fushi Tarazu Gene

A. FTZ-F1, a Transcriptional Activator of Ftz Gene (G. Lavorgna)

We have previously cloned the *ftz* transcriptional activator FTZ-F1 and showed its similarity to the steroid receptor superfamily.

The structure of FTZ-F1 during development is now being studied and we have identified at least three different mRNA species. The first one (corresponding at the one previously cloned, is detectable at 0-4 hour of development and is maternally and zygotically expressed) shares with the other two (detectable starting from 14 hours of development) the putative ligand binding domain and the putative DNA-binding domain, but differs in the upstream region where no known sequence motif has been identified. Studies are under way to determine the functional differences between the three different forms.

Additionally, the *Drosophila* FTZ-F1 gene was expressed at high levels in *E. coli* in a soluble form. The recombinant protein was found to be highly active for binding to DNA specifically. Purified recombinant FTZ-F1 was used for raising rabbit polyclonal antibodies. The antibodies show reactivity with native FTZ-F1 protein in a gel shift assay and will be used to detect the presence of FTZ-F1 protein "in situ" on embryos.

We have also started to approach the problem of eliminating FTZ-F1 function in vivo and are currently following two experimental plans:

- i) Southern blot analysis of strains carrying deletions in the 75 region of the salivary gland map (the locus to which FTZ-F1 maps).
- ii) Injection of antisense FTZ-F1 oligos during early stages of development in order to inhibit the maternal FTZ-F1 contribution in the embryo.
- iii) Transformation of *Drosophila* embryos with the FTZ-F1 gene under heat shock control.

B. FTZ-F2 (tramtrack), a Transcriptional Repressor of the Ftz Gene (J. L. Brown)

FTZ-F2 is a group of four activities that bind to the same sequence in a 69 bp region of the *ftz* zebra regulatory element. Mutations of the 69 bp region that abolish FTZ-F2 binding cause a derepression of *ftz-lacZ* expression in preblastoderm embryos as early as the 3rd nuclear division cycle. The results suggest FTZ-F2 functions as a transcriptional repressor of the FTZ gene during the initial nuclear division cycles. A putative cDNA clone encoding FTZ-F2 has been isolated, sequenced and found to be identical to the tramtrack protein, a zinc finger protein previously cloned by A. Travers and colleagues in Cambridge, England. In order to rigorously relate the FTZ-F2 protein activities to the tramtrack protein, we have expressed the open reading frame of our cDNA clone in *E. coli* and raised polyclonal antibodies to the recombinant protein. We have found that the antibodies do react with all the FTZ-F2 activities, thus confirming that FTZ-F2 and tramtrack are closely related, if not identical. The antibodies are currently being used to study the distribution of FTZ-F2 during

Drosophila development. To address the in vivo function of FTZ-F2 we are constructing transgenic flies carrying FTZ-F2 under the control of the heat shock promoter.

Publications:

Clos J, Westwood JT, Becker P, Wilson S, Lambert K, Wu C. Molecular cloning and expression of a hexameric Drosophila heat shock factor subject to negative regulation, Cell 1990;63:1085-97.

Zimarino V, Wilson S, Wu C. Antibody-mediated activation of Drosophila heat shock factor in vitro, Science 1990;249:546-9.

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.

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, in press.

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, in press.

Lis J, Wu C. Heat shock factor. In: Yamamoto KR, McKnight SL, eds. Transcriptional regulation. New York: Cold Spring Harbor Press, 1991, in press.

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, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05264-10 LB

PERIOD COVERED
October 1, 1990 to September 30, 1991

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

Intracisternal A-Particle (IAP) and Growth Factor Genes in Mouse Myelomas

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)

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Biosynthesis Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

Growth and differentiation of B-cells to plasma cells involve regulation by a variety of interleukins. Development of plasma cell neoplasias (myelomas) requires, in addition, activation of several oncogenes. Rearrangement of the interleukin 6 (IL-6) gene, and its subsequent constitutive expression in a myeloma, led to the suggestion that IL-6 may be involved in development of certain myelomas by an autocrine growth mechanism. Therefore, we analyzed genomic DNA from a collection of early generation myelomas to determine whether any of these had rearrangements in the IL-6 gene. A total of 55 myeloma DNAs were analyzed, with 40 of these from myelomas at generation 3 or earlier. Southern analysis of DNA cut with *EcoRI* and probed with an IL-6 probe would detect rearrangements at both the 5' or 3' ends of the gene. No differences were found in the genomic IL-6 DNA in the myelomas compared with that in the germline DNA. Thus it is unlikely that continuous deregulated expression of IL-6 due to a gene rearrangement plays a major role in generation of myelomas in the mouse.

IAP genes expressed in LPS stimulated B-cells of the BALB/c mouse have been shown to represent a restricted set of elements which can be distinguished by specific sequences in the R regions of the LTR (see Kuff Annual Report 91).

Project Description

Objectives:

1. To determine whether growth factor gene rearrangements play a role in development and/or progression of mouse myelomas.
2. To characterize the IAP elements expressed in a myeloma.

Major Findings:

Loss of growth factor dependence, along with activation of oncogenes, has been proposed to be a common early step in neoplastic transformation of hemopoietic cells. In many cases, growth factor independence has resulted from autocrine synthesis of factor after rearrangement of the gene due to insertional mutagenesis. In numerous examples involving cells of the myeloid lineage, the inserted DNA was shown to be an intracisternal A-particle (IAP) genetic element. Insertions have occurred into the granulocyte monocyte colony stimulating factor (GM-CSF) gene, the IL-3 gene, the IL-5 gene, and the Hox-2.4 gene. Rearrangements involving IAP genetic elements have also been observed in MOPC-11 and MOPC-21 myeloma cell lines into the IL-6 gene and IL-6 receptor gene, respectively. Continuous deregulated expression of IL-6 had been proposed to play a role in generating myelomas. If that were the case, such rearrangements would be expected to occur early in the development of the tumors. Since we have evidence that IAP genes are expressed in early stages of myeloma development (see Kuff, AR91), and it is known that IAP elements can transpose by retrotransposition in cells that express them, growth factor rearrangements, some possibly involving IAP elements, might be common in the mouse myeloma system.

Southern Blot Analysis of Myeloma DNAs

Myeloma DNAs (provided by Dr. Michael Potter, NCI) were digested with EcoRI, fractionated on agarose gels, and blotted to nylon membranes. This restriction enzyme cut permits detection of rearrangements at both the 5' and 3' ends of a variety of growth factor genes. Fifty-five myeloma samples were analyzed as follows: 3 G0, 7 G1, 24 G2, 6 G3, 3 G4, 1 G6, 1 G8, 10 >G10 (G=generation). Probing of the blots with an IL-6 cDNA clone (obtained from DNAX, Inc.), showed no differences in the pattern of fragments in myeloma and germline DNA. We conclude that the rearrangement of the IL-6 gene seen in the MOPC-21 myeloma is not a general event common to all myelomas.

Characterization of IAP Elements Expressed in a Myeloma

IAP genes expressed in LPS stimulated B-cells of the BALB/c mouse have been shown to represent a restricted set of elements which can be distinguished by specific sequences in the R regions of the LTR (see Kuff Annual Report 91). Since myelomas generally express high levels of IAP RNA and can be considered the transformed counterpart of B-cells, we would like to determine whether the same IAP elements are expressed at higher levels after transformation of the cells, or whether new IAP genes are activated. Using a MOPC-11 myeloma cDNA library in lambda phage gt10 (obtained from Michael Kuehle, NIMC), 50 phage

plaques reacting with a general IAP LTR probe were isolated. Inserts, with an average size of 1.3 kb, were amplified from phage suspensions by PCR with gt10 primers, and blotted to filters for hybridization. These will be screened with oligonucleotide probes that detect the subsets of IAP elements expressed in B-cells. Results from this sample of cDNAs thus far show that one IAP gene class expressed in B-cells is not expressed in the myeloma, while a second class is expressed. Clones not falling into the previously characterized classes will be sequenced. This analysis should also permit us to determine whether the IAP element involved in the IL-6 gene transposition in this myeloma represents a major or minor IAP transcript.

IAP Gene Expression in Pancreatic β -Cells

In the last report we described cloning of IAP cDNAs from C57BL/Ks (diabetes susceptible) mouse pancreatic β -cells. Two oligonucleotide probes based on sequences in these clones that differed from other IAP clones detect the same restriction fragment length polymorphisms in BamHI cut genomic DNA of C57BL/Ks mouse. Attempts to clone these fragments from size fractionated DNA have thus far been unsuccessful.

Publication:

Lueders KK. Genomic organization and expression of endogenous retrovirus-like elements in cultured rodent cells, *Biologicals* 1991;19:1-7.

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

PROJECT NUMBER

Z01 CB 05265-09 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Y. Wu	Visiting Fellow	LB	NCI
Y. Yang	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

3.6

PROFESSIONAL:

3.6

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

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. Most of our work is done with digitonin-permeabilized cells. The release of catecholamines from these permeabilized cells is both Ca^{2+} and ATP dependent.

Protein phosphatases and phosphatase inhibitors were used to examine the role of protein phosphorylation in the regulation of secretion. Our results suggest that norepinephrine secretion by both PC12 cells and chromaffin cells is regulated by a Ca^{2+} -stimulated phosphorylation.

Digitonin-permeabilized chromaffin cells are used to identify proteins involved in the secretory response. Incubation of these permeabilized cells results in a loss of Ca^{2+} -dependent secretion. The addition of cytosolic proteins restores this secretory activity. We have fractionated cytosolic extracts and demonstrated that more than one protein is required for Ca^{2+} -dependent secretion. We have purified one of these proteins and are in the process of characterizing it. Also in contrast to a published report, we have shown that calpactin is not involved in this restoration.

Secretion of norepinephrine by digitonin-permeabilized PC12 cells can also be stimulated by the addition of GTPYS. We are continuing to try to determine the mechanism by which GTPYS induces secretion in these cells. Several aspects of this stimulation suggest that the protein responsible for this stimulation might be a low molecular GTP-binding protein rather than a heterotrimeric GTP-binding protein.

Project DescriptionRegulation of SecretionObjectives:

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

Major Findings:

To study the mechanism of secretion and its regulation by Ca^{2+} , we have been using both primary cultures of bovine adrenal chromaffin cells and PC12 cells, an established cell line isolated from a rat pheochromocytoma. Stimulation of bovine chromaffin cells and PC12 cells by nicotine or K^{+} -depolarization results in the Ca^{2+} -dependent secretion of catecholamines. Most of our work has been done 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 from these permeabilized cells is both Ca^{2+} - and ATP-dependent and occurs by exocytosis. Permeabilization of the plasma membrane gives access to the cytoplasm and allows one to investigate the mechanism of secretion. While permeabilized bovine chromaffin cells and PC12 cells have similar secretory activities, there are significant differences. For example, while permeabilized bovine chromaffin cells rapidly lose the ability to secrete catecholamines, permeabilized PC12 cells retain nearly full secretory activity even after a one hour incubation.

We have previously presented evidence that secretion in permeabilized PC12 cells is regulated by a Ca^{2+} -stimulated phosphorylation of some as yet unidentified protein. According to our proposal when permeabilized PC12 cells are incubated with ATP in the absence of Ca^{2+} , some phosphorylation occurs, but the activities of Ca^{2+} -independent phosphatases are sufficient to keep the protein dephosphorylated, and there is little secretion. The addition of Ca^{2+} activates a kinase (or Ca^{2+} binding to a target protein increases its rate of phosphorylation by a Ca^{2+} -independent kinase) resulting in increased levels of phosphorylation and, consequently, increased levels of norepinephrine secretion. We have now examined whether norepinephrine secretion by bovine chromaffin cells is also regulated by a Ca^{2+} -stimulated phosphorylation.

Addition of okadaic acid, potent inhibitor of type 1 and type 2A protein phosphatases, or 1-naphthylphosphate, a more general phosphatase inhibitor, to digitonin-permeabilized chromaffin cells caused about a 100% increase in the amount of norepinephrine secreted in the absence of Ca^{2+} without affecting the amount secreted in the presence of saturating Ca^{2+} . This stimulation of

norepinephrine secretion by protein phosphatase inhibitors suggests that in the absence of Ca^{2+} there is a slow rate phosphorylation and that this phosphorylation triggers secretion. Addition of an exogenous type 2A protein phosphatase caused almost a 50% decrease in Ca^{2+} -dependent norepinephrine secretion. Thus, the amounts of norepinephrine released both in the absence of Ca^{2+} and in the presence of Ca^{2+} appear to depend upon the level of protein phosphorylation. These data are consistent with secretion in bovine chromaffin cells being induced by a Ca^{2+} -stimulated phosphorylation.

These results with digitonin-permeabilized chromaffin cells are similar to those we reported previously with digitonin-permeabilized PC12 cells. One significant difference is the effect of okadaic acid. Unlike the results obtained with chromaffin cells, the addition of okadaic acid to digitonin-permeabilized PC12 cells had no effect on the amount of norepinephrine released in the absence of Ca^{2+} . While two general phosphatase inhibitors, 1-naphthylphosphate and sodium pyrophosphate, did increase basal secretion in permeabilized PC12 cells, the greater specificity of okadaic acid for protein phosphatases makes one more confident that inhibition of protein phosphatases can induce secretion.

Incubation of digitonin-permeabilized bovine chromaffin cells results in a loss of Ca^{2+} -dependent norepinephrine secretion. We have found that the addition of a cytosolic extract to these depleted cells restores Ca^{2+} -dependent norepinephrine secretion. As digestion of this extract with trypsin results in a loss of its ability to restore secretion, proteins present in this extract are required for restoring secretory activity. While we were in the process of trying to isolate the protein or proteins which are responsible for this restoration, Burgoyne and coworkers reported that the addition of calpactin to digitonin-permeabilized chromaffin cells prevented the loss of secretory activity and suggested that calpactin was the Ca^{2+} -receptor protein involved in triggering secretion. Calpactin is a Ca^{2+} -dependent phospholipid and actin binding protein. However, calpactin only had an effect when secretion was measured in saturating Ca^{2+} . Secretion in subsaturating Ca^{2+} was not affected by the addition of calpactin. Because of this report, we examined the effect of calpactin on secretion. An anti-calpactin antibody column was used to deplete the cytosolic extract of calpactin. This calpactin-depleted extract was just as effective as the control extract in preventing the loss of secretory activity. Also in contrast to the results reported by Burgoyne, we found that the addition of calpactin by itself had little or no effect on norepinephrine secretion. Thus, cytosolic proteins other than calpactin appear to be responsible for preventing the loss of secretory activity.

Because calpactin does not appear to be the protein responsible for restoring secretion, we have continued to try to isolate the proteins responsible for this restoration. Our initial attempts to fractionate cytosolic proteins failed to identify a single active fraction, rather several fractions were found to contain partial activity. Mixing experiments indicated that these fractions contain different proteins and that more than one cytosolic protein is required to restore secretion to digitonin-permeabilized chromaffin cells. From one of these fractions we have purified a protein which can partially prevent the loss of secretory activity. It appears to be a dimer made up of 30,000 dalton subunits. We are in the process of characterizing this protein.

Secretion of norepinephrine by digitonin-permeabilized PC12 cells can also be stimulated by the addition of GTPYS or GMP-PNP but not GTP. While secretion in the presence of saturating Ca^{2+} is not affected by GTPYS, secretion in the absence of Ca^{2+} is stimulated 2 to 3 fold by the addition of GTPYS. This stimulation by GTPYS 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 GTPYS-stimulated norepinephrine release, and cholera and pertussis toxins have little or no effect on GTPYS-stimulated norepinephrine secretion. We are continuing to try to determine the mechanism by which GTPYS induces secretion in these cells. Unlike Ca^{2+} -stimulated norepinephrine release, there is a delay between the time of addition of GTPYS and stimulation of norepinephrine release. Preincubation of the permeabilized cells in the absence of Mg^{2+} eliminated this lag and increased the initial rate of GTPYS-stimulated norepinephrine secretion. This suggests that the rate of GDP dissociation from the GTP-binding protein responsible for this release is faster in the absence of Mg^{2+} than in its presence. While an equimolar concentration of GTP gives 50% inhibition of GTPYS-stimulated release, 100-fold excesses of ITP, ATP, UTP, and CTP gave no inhibition of GTPYS-stimulated release. Both the inability of ITP to inhibit GTPYS-stimulated secretion and the increase in GTPYS-stimulated secretion caused by preincubation in the absence of Mg^{2+} indicate that some of the properties of the GTP-binding protein responsible for this stimulation are more like those of the low molecular GTP-binding proteins rap 1 and ras than those of a heterotrimeric G-protein.

Publications:

Wagner PD, Vu ND. Regulation of norepinephrine secretion in permeabilized PC12 cells by Ca^{2+} -stimulated phosphorylation: effects of protein phosphatases and phosphatase inhibitors, *J Biol Chem* 1990;265:10352-7.

Carroll AG, Rhoads AR, Wagner PD. Hydrolysis-resistant GTP analogs stimulate catecholamine release from digitonin-permeabilized PC12 cells, *J Neurochem* 1990;55:930-6.

Wu YN, Wagner PD. Calpactin-depleted cytosolic proteins restore Ca^{2+} -dependent secretion to digitonin-permeabilized bovine chromaffin cells, *FEBS Lett* 1991;282:197-9.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 CB 05267-07 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanisms of Plasmid Maintenance

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

M. Yarmolinsky	Chief, Microbial Genetics and Biochemistry Section	LB	NCI
D. Chattoraj	Microbiologist	LB	NCI
G. Mukhopadhyay	Visiting Fellow	LB	NCI
S. Sozhamannan	Visiting Fellow	LB	NCI
P. Papp	Visiting Fellow	LB	NCI
M. Lobočka	Visiting Fellow	LB	NCI

COOPERATING UNITS (if any)

Dr. R. Rosenfeld, Hadassah Medical School, Jerusalem, Israel; Dr. H. Lehnerr, Biozentrum, Basel, Switzerland; Dr. T. Schneider, NCI, FCRCD, Laboratory of Mathematical Biology.

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SECTION

Microbial Genetics and Biochemistry Section

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TOTAL MAN-YEARS:

6.0

PROFESSIONAL:

6.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

The remarkable stability of the low copy number plasmid P1 results from a combination of mechanisms under continuing study: replication control, active partition, and plasmid addiction. Previous work had shown that plasmid replicons with shared requirements for particular bacterial proteins (DnaA, Dam) can exhibit surprising differences in the manner in which these proteins are used. Work performed in the period of the present report supports the extension of this caveat to include the three proteins DnaJ, DnaK, GrpE. These heat shock proteins appear to play a simpler role in P1 replication and one more accessible to analysis than that previously established for their role in the replication of phage lambda. The separate functions of the P1 protein, RepA, in initiation of DNA synthesis and in mediation of copy number control by inca are being dissected with in vitro techniques. Two-dimensional gel electrophoresis assays provide direct information about DNA conformation. Sequence determinations of RepA binding sites generated by "dirty bottle" synthesis and subjected to informational analysis provide indications that conserved regions of DNA sequence in binding sites may serve different functions. In addition to mechanistic studies of protein-DNA interactions, studies of a "back-to-basics" kind are being undertaken. These test the range of validity of the functional independence of active partition and replication. In particular, newly recognized context effects that can render the P1 partition system destabilizing are being explored. The nature of an addiction system in P1 which appears unlike those found associated with other plasmids is also being studied.

Project DescriptionObjectives:

The objective of the work reported here is to understand, in molecular detail, the several mechanisms that enable Pl prophage to be stably inherited as a plasmid. This objective is pursued with the conviction that information obtained about this microcosm is relevant to the understanding of such fundamental biological processes as the cell cycle (ref. 3).

A. Role of Heat Shock Proteins in Pl Replication.

Binding of the Pl replication initiator protein, RepA, to its target DNA iterons, is reduced in cells deficient in any one of the heat shock proteins DnaJ, DnaK and GrpE. Overproduction of RepA in HSP mutants (inhibitory to Pl replication in wild type bacteria [ref. 1]) overcomes both the binding defect (ref. 2) and the associated reduction in the replication proficiency of Pl. Mutations in repA that confer increased iteron affinity (as judged by in vivo and in vitro assays) have been isolated and characterized by Sozhamannan, Mukhopadhyay, Papp and Chatteraj. Their work suggests that origin-RepA binding is the only step in Pl replication in which HSPs play a role. (Compare lambda phage replication for which HSPs act to dissociate a host-encoded helicase from a protein complex at the origin.) The availability of purified mutant protein should facilitate an understanding of the nature of the RepA protein alteration induced by HSPs. An HSP-induced decrease in RepA binding to non-specific sites on DNA suggests that the HSP effect on specific binding may be largely due to release of RepA from sites that compete with the iterons. This hypothesis is being evaluated.

B. Role of Histone-like Bacterial Protein H1 in Plasmid Replication.

Like IHF and HU, H1 is an abundant small DNA-binding protein that acts as a global regulator. Evidence from other laboratories for the importance of IHF in the replication of pSC101 and R6K and of HU in the replication of Pl suggested a possible role for H1 in plasmid replication. Chatteraj confirmed that HU plays an essential role in Pl plasmid replication, but found that plasmids poriC, Pl, F, pSC101, R6K, and pBR322 are not sensitive to an H1 defect.

C. Wrapping of Pl Origin DNA Around RepA.

The finding of an increase in DNA bending upon successive binding of RepA to the five iterons of the Pl origin was mentioned in the previous annual report. The magnitude of this bend suggested that the origin DNA might wrap approximately once around the RepA complex. The prediction was verified by Mukhopadhyay and Chatteraj using closed circular DNA. RepA binding absorbed about one positive superhelical turn of DNA. How this wrapped structure affect neighboring DNA remains to be determined.

D. Informational Analysis of RepA Binding Sites. (in collaboration with T. Schneider).

The distribution of conserved sequences in the 14 iterons to which RepA binds is tripartite. The two outside patches include bases that contact RepA through two consecutive major grooves as determined by dimethyl sulfate footprinting. The centrally conserved patch should then lie in a minor groove on the face of DNA which contacts RepA. If the tripartite distribution was required to specify binding alone, it would be inconsistent with the specification being entirely through major groove contacts on one "face" of the helix, as normally found in prokaryotes. The central patch was found by Papp and Chatteraj to be absent from among a large number of synthesized variant iterons that were selected for retention of affinity for RepA. The possibility is being explored that the central patch is used by RepA in a separate binding mode that provides for RepA-mediated iteron pairing, as postulated by Chatteraj to be involved in Pl copy number control.

E. Addictive Functions Encoded by Pl (in collaboration with H. Lehnerr).

Previous studies by Maguin and Yarmolinsky identified a system that confers severe withdrawal symptoms on bacteria from which Pl is lost. The region of the Pl genome that encodes this addiction system has now been localized to within one kb of DNA. The encompassed small open reading frames show no obvious homology to those of other plasmids (F, R1) that are known to encode proteins with analogous function.

F. Characterization of the Partition Complex at parS, the Plasmid "Centromere".

Studies from this laboratory on interactions among the Pl-encoded partition protein ParB, the host-encoded accessory protein IHF (integration host factor), and the Pl parS site to which these proteins bind cooperatively, are the subject of a report in press (ref. 4). This work will be continued by Funnell at the University of Toronto.

G. Partition System-Mediated Plasmid Destabilization.

The two proteins ParA and ParB that, together with the Pl centromere site (parS), comprise the partition system of Pl, stabilize various plasmids if they bear a parS site. However, such plasmids are destabilized if ParB is present in excess. Lobočka and Yarmolinsky find that parS inserted in either orientation into the thermosensitive pSC101-derived vector, pGB2ts, when supplied with ParA and ParB in proportions that we expected to be stabilizing, drastically destabilized this otherwise stable plasmid. Previous unsuccessful attempts in this laboratory to clone a complete Pl Par system into oriC plasmids may have been due to similar effects. The requirements for the destabilization observed are under study by selective alteration of components of the system including analysis of mutants that restore stability in the presence of both Par proteins.

H. Partitioning in the Absence of Replication (in collaboration with R. Rosenfeld).

An assay developed by Treptow and Yarmolinsky for Pl Par function in the absence of replication of the partitioned DNA has provided positive, but preliminary, evidence that the Pl partition system can operate effectively on unreplicated DNA (ref. 3). It was in the course of attempts to improve the assay that the results described under (G) were obtained. Improvements in the assay system will continue to be explored.

Publications:

Muraiso K, Mukhopadhyay G, Chattoraj D. Location of a Pl plasmid replication inhibitor determinant within the initiator gene, *J. Bact* 1990;172:4441-7.

Tilly K, Sozhamannan S, Yarmolinsky M. Participation of the Escherichia coli heat shock proteins DnaJ, DnaK, and GrpE in autorepression of the Pl plasmid repA promoter, *The New Biologist* 1990;2:812-7.

Yarmolinsky MB. Le cell cycle (meeting review), *The New Biologist* 1991; 3:50-6 (outside activity).

Funnell B. The Pl plasmid partition complex at parS: the influence of Escherichia coli integration host factor and of substrate topology, *J Biol Chem* 1991, in press.

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

PROJECT NUMBER

Z01 CB 05268-04 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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T-C. Wu	Chemist	LB	NCI
C. Goyon	Visiting Fellow	LB	NCI
M. Daly	IRTA Fellow	LB	NCI

COOPERATING UNITS (if any)

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SECTION

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INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.4

PROFESSIONAL:

3.4

OTHER:

-

CHECK APPROPRIATE BOX(ES)

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

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* and the means by which yeast controls meiotic recombination. We have focused on what is thought to be the initiating event in meiotic recombination, the formation of meiosis-specific double-strand DNA breaks, and have begun to determine that factors that control the level and position of these breaks. In addition, we have continued the analysis of several short regions of the yeast genome that contain sequences that stimulate meiotic recombination, and have shown that these elements also stimulate the formation of double-strand DNA breaks during meiosis.

Project DescriptionObjectives:

Our ultimate objective is an understanding of the molecular mechanism of meiotic recombination, using the yeast Saccharomyces cerevisiae as a model system. We are engaged in a series of experiments designed to describe, at the molecular level, the entire process of meiotic recombination, from initiating lesions, through intermediate structures, to the production of mature recombinant products. As part of this study, we are examining the timing of various molecular events during meiosis. As another part of this study, we are examining the means by which the level of meiotic recombination is controlled in different parts of the yeast genome, including the isolation and characterization of elements that promote meiotic recombination.

Major Techniques Employed and Major Findings:A. Timing of Recombination Events During Meiosis

We have continued experiments that use physical methods to detect recombined molecules and recombination intermediates. Dr. Christophe Goyon had previously determined the time of appearance of double-strand DNA breaks (thought to initiate meiotic recombination), of heteroduplex DNA, and of mature recombined DNA molecules. His results indicated that, although the initial events in meiotic recombination (formation of double-strand DNA breaks) occur early in meiosis, stable intermediates containing heteroduplex DNA are not formed until late in meiosis, some 1-2 hours later. Dr. Goyon has since refined his experimental approach to allow isolation and detection DNA molecules that contain short (about 100 bp) regions of hybrid DNA; the same gap between double-strand break formation and appearance of heteroduplex-containing molecules was observed. We are currently developing techniques, including the detection of intermediate structures that contain three or four-stranded DNA molecules, to examine events that occur during this gap.

B. Factors that Control the Position and Level of Meiosis-Induced Double-Strand DNA Breaks

We have shown that the formation of double-strand DNA breaks during meiosis does not involve simply the recognition and cutting of a short nucleotide sequence. Initial evidence for this came from an examination by Christophe Goyon of the meiotic behavior of the normal ARG4 locus and of ARG4 sequences inserted at another place in the yeast genome. At the normal ARG4 locus, a meiosis-induced double-strand break occurs in the ARG4 promoter region. However, no double-strand break occurred in this region when ARG4 was inserted at the MAT locus in the context of a pBR322-based plasmid. Instead, a prominent break occurred at the junction of ARG4 and pBR322 sequences, 1.2 kb upstream of the original ARG4 break site. Carol Wu has extended this study to an examination of the meiotic behavior of this ARG4-pBR322 construct inserted at other locations in the yeast genome. She has shown that the level of meiotic recombination in ARG4 can vary significantly, and is

determined primarily by the locus at which ARG4 is inserted. Inserts that produce high levels of ARG4 recombinants display the highest amounts of double-strand break at the ARG4-pBR322 junction; inserts with lower levels of recombination display correspondingly lower levels of the same double-strand break. Thus, levels of meiotic recombination within the ARG4 gene and levels of a double-strand break just outside this gene are determined, at least in part, by the same factors. However, the role that this break plays in initiating meiotic recombination in ARG4 has been called into question. Deletion of the site of this break resulted in a substantial reduction in the level of breaks observed during meiosis, but did not reduce recombination within the ARG4 insert. We are currently engaged in an examination of the role that chromosome and chromatin structure play in determining the level and position of meiosis-induced double-strand breaks, and are also performing genetic and physical experiments to clarify the relationship between these breaks and recombination in ARG4.

C. Sequences That Modulate the Level of Meiotic Recombination

Michael Daly has isolated a number of 2-3 kb fragments of yeast DNA that promote meiotic recombination. He has focused attention on two fragments that appear to contain portable stimulators of meiotic recombination. These fragments stimulate meiotic recombination when inserted at several loci in the yeast genome, and act in a meiosis-specific manner (i.e. they do not stimulate mitotic recombination). They promote recombination when heterozygous, and act in cis to promote gene conversion at sites in sequences flanking the element. In addition, these elements stimulate the formation of double-strand breaks during meiosis. These breaks are not located in the fragment itself, but instead occur in surrounding sequences. Thus, it appears that these cloned fragments contain some sort of "enhancer" element which can act at a distance to promote meiotic recombination and formation of double-strand breaks. Dr. Daly is currently engaged in further characterization of the nature of the elements contained in these fragments, both by subcloning and by DNA sequencing. In addition, he is determining the distance- and orientation-dependence of their action.

Publications:

Lichten M, Goyon C, Schultes NP, Treco D, Szostack JW, Nicolas A. Detection of heteroduplex DNA molecules among the products of Saccharomyces cerevisiae meiosis, Proc Natl Acad Sci USA 1990;87:7653-7.

Haber JE, Leung WY, Borts RH, Lichten M. The frequency of meiotic recombination in yeast is independent of the number and position of homologous donor sequences: implications for chromosome pairing, Proc Natl Acad Sci USA 1991;88:1120-4.

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

PROJECT NUMBER

Z01 CB 05269-04 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

DNA Synthesis in Mammalian Cells: Studies of Nucleic Acid Binding Proteins

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

S. H. Wilson	Chief, Nucleic Acid Enzymology Section	LB	NCI
A. Kumar	Expert	LB	NCI

COOPERATING UNITS (if any)

F. Robey, NIH; E. Ackerman, NIH; K. Seamon, FDA; K. Williams, Yale; R. Karpel, University of Maryland; F. Cobianchi, CNR, Pavia, Italy; B. Kay, University of North Carolina; A. Gronenbonn, NIH; M. Clore, NIH, S. Haynes, NIH.

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Nucleic Acid Enzymology Section

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National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

Physical biochemical studies of a single-stranded nucleic acid binding protein termed Al were conducted. The protein binds cooperatively to either RNA or DNA and the full length protein binds much tighter than a truncated Al protein lacking the glycine-rich COOH-terminal domain (residues 185-319). Our studies suggest the mechanism of Al binding is, in some respects, similar to that of two well-known prokaryotic ssDNA binding proteins: Binding involves charge-charge interactions and close approach of aromatic amino acids to nucleotide bases. Our findings indicate that both the NH₂-terminal and COOH-terminal domains of the intact Al protein make significant contributions to the overall free energy of binding to nucleic acids. Both the COOH-terminal fragment and intact protein have strand annealing activity for both RNA and ssDNA.

Project DescriptionObjectives:

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

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

Major Findings:

A. Structure-Function Studies of the Single-Stranded Nucleic Acid Binding Protein: Mammalian hnRNP Protein A1

1. Immediately following transcription, heterogeneous nuclear RNA (hnRNP) becomes associated with a group of proteins to form a ribonucleoprotein (hnRNP) complex that is believed to function in the processing, stabilization and transport of unique sequence transcripts. Earlier, we cloned a cDNA for the A1 hnRNP protein and then overproduced the protein in E. coli. We now are using a variety of physicochemical approaches to identify functional domains in the protein. Sequencing revealed that the first 184 amino acids in A1 contain an internal repeat such that when residues 3-93 are aligned with 94-184, 33% of the amino acids are identical. Limited proteolysis and photochemical cross-linking experiments indicate that the internal repeats in A1 correspond to two independent nucleic acid binding domains.

With a view toward understanding structure-function relationships of a protein component of the eukaryotic heterogeneous ribonucleoprotein complex, we have studied the interactions of recombinant A1 protein, its N-terminal two-thirds (UPl), and C-terminal domain with the fluorescent polynucleotide, poly (ethenoadenylic acid), poly(ϵ A). The overall binding affinity of A1 for poly(ϵ A) is greater than that of UPl. This can be partly, but not entirely explained by the existence of cooperativity in Al-nucleic acid interactions: The cooperativity parameter, w , for Al-poly (ϵ A) interaction is about 30, whereas UPl binds nucleic acids non-cooperatively ($w=1$). Binding experiments with the C-terminal domain show that this polypeptide is capable of binding to single-stranded nucleic acids, and these results suggest that intact A1 has at least two potential binding regions for nucleic acids: the N-terminal domain and the C-terminal domain.

2. The structure of A1 has been investigated using fluorescence emission properties of tryptophan and tyrosine residues. Both steady-state fluorescence and dynamic fluorescence have been used. The results indicate that the single tryptophan residue in A1, which incidentally is located in the N-terminal domain, is on the surface of the protein and is

freely exposed to solvent. Upon binding to a polynucleotide lattice, fluorescence properties of this tryptophan residue are essentially unchanged, indicating that the residue does not participate in the nucleic acid binding function. In contrast, the tyrosine residues in A1 (12 are present) are strongly quenched in fluorescence emission upon nucleic acid binding. The results are consistent with a model where tyrosine residues interface directly with nucleic acid base residues to achieve a portion of the overall free energy of binding.

3. The A1 protein and its C-terminal domain fragment of 12 kDa are capable of annealing activity for base-pair complimentary single-stranded polynucleotides. This activity is similar to that exhibited by the well known E. coli recombination protein termed recA. However, in the case of A1, annealing activity also is seen with single-stranded RNA polynucleotides.
4. The N-terminal domain of A1 is a globular single-stranded nucleic acid binding protein of about 22,000 daltons. In order to study the three-dimensional structure of this protein by NMR spectroscopy, samples were prepared with ¹³C and ¹⁵N substitutions. Analysis of these samples is under way in the laboratory of Drs. Clore and Gronenborn.

Publications

Kay BK, Sawhney RK, Wilson SH. Potential for two isoforms of the A1 ribonucleoprotein in Xenopus laevis, Proc Natl Acad Sci USA 1990;87:1367-71.

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Nadler SG, Merrill BM, Roberts WJ, Keating KM, Lisbin MJ, Barnett SF, Wilson SH, Williams KR. Interactions of the A1 heterogeneous nuclear ribonucleoprotein and its proteolytic derivative, UPI, with RNA and DNA, Biochemistry 1991;30:2968-76.

Wilson SH. Mammalian hnRNP complex protein A1 and mechanism of single-stranded nucleic acid binding (Review). In: Wilson SH, ed; Cancer biology and biosynthesis. Caldwell, NJ: The Telford Press, 1991;58-9.

Casas-Finet JR, Karpel RL, Maki AH, Kumar A, Wilson SH. Physical studies of the tyrosine and tryptophan residues in mammalian heteronuclear ribonucleoprotein A1 and of its constituent domains, J Mol Biol, in press.

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

PROJECT NUMBER

Z01 CB 05270-04 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

DNA Synthesis in Mammalian Cells: Mechanism of HIV Reverse Transcriptase

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

S. H. Wilson	Chief, Nucleic Acid Enzymology Section	LB	NCI
J. Abbotts	NRC Fellow	LB	NCI
P. Becerra	Expert	LB	NCI
A. Kumar	Expert	LB	NCI
S. Widen	Staff Fellow	LB	NCI
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COOPERATING UNITS (if any) S. Boucage, FDA; S. Hughes, NCI-FCRF; S. Shiloach, NIH; D. Jerina, NIH; T. Kunkel, NIH; A. Gronenborn, NIH; S. Stahl, NIH; D. Davies, NIH, J. MacMahon, NCI-FCRF; R. Suhadolnik, Temple Univ.; D. Hatfield, NIH; W. Egan, FDA; M. Lewis, NIH.

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Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews 40% of total effort

B

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

We continued detailed biochemical studies on mechanisms of action of mammalian DNA replication proteins. Steady-state kinetic analysis of the HIV reverse transcriptase confirmed an overall kinetic scheme for the reverse transcriptase mechanism proposed earlier, and properties of the first steps in the proposed reaction pathway were studied. A DNA segment containing the coding region for this enzyme was subcloned into an expression vector, and the enzyme and several domain fragments were overproduced from *E. coli* and purified. These recombinant enzymes and proteins are under study. The enzyme-primer binding pocket was localized by photochemical cross-linking with primer analogues, oligo d(T)₈ and oligo d(T)₁₆. In other experiments, the protein was altered by site-directed mutagenesis in order to understand mechanistic aspects of primer recognition. The proposed dNTP binding pocket in the enzyme was manipulated by site-directed mutagenesis. A collection of ~20 mutants has already been obtained, and the collection will be substantially enlarged during the next year. Binding between the enzyme and its substrates is being examined using equilibrium binding techniques based upon fluorescent reporters in the substrates. Another area of investigation is RT subunit interaction. HIV-1 RT works in the cell as a complex of two protein molecules. Compounds that block interaction between these two proteins may prove to be highly specific inhibitors of the RT. We have identified a small region in the RT that is responsible for this protein:protein interaction.

Project Description

Objectives:

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

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

Major Findings:

A. Structure-Function Relationships of HIV Reverse Transcriptase

1. A recombinant E. coli plasmid, pRC-RT, containing the coding region for HIV-1 RT was constructed, and a method was obtained for overexpression and purification of the enzyme. In the vector for expression, the precise RT coding region of HXB2 proviral DNA is flanked by start and stop codons, and expression is driven by the temperature-inducible λ P_L promoter. The RT polypeptide of 64,484 Da (termed p66) is expressed at the level of 10% total cell protein after 2 h of induction. The RT is solubilized and purified as a p66 polypeptide or as a mixture of p66 and a 51,000-M_r peptide. p66 RT, like virion-derived HIV RT, is processive, yet exhibits a slightly different termination pattern than natural RT on an M13 DNA template. Efforts are under way to obtain crystal structure information on this enzyme (collaboration with D. Davies). This type of information will greatly facilitate design of specific substrate analogue inhibitors, more potent than AZT and ddI.

After achieving satisfactory expression of the full-length p66 reverse transcriptase, we created vectors for expression of four individual segments of the p66 polypeptide. These segments are: First, a 51,000 Da peptide, representing a C-terminal truncation of the p66 polypeptide; and, second, three peptides representing consecutive N-terminal, control, and C-terminal segments, respectively, of p66; these segments are termed p29, p20, and p15, respectively. p15, which corresponds to the ribonuclease H domain of reverse transcriptase has been purified in large quantities, crystallized, and is currently under study for solution of its 3-dimensional structure. This ribonuclease H domain peptide, incidentally, is devoid of enzyme activity and of substrate binding capacity, but does exist in solution as a tightly folded globular protein. Its structure in solution resembles that of E. coli ribonuclease H, as revealed by preliminary NMR analysis (collaboration with A. Gronenborn).

B Mapping of a Dimerization Domain in the HIV Reverse Transcriptase

1. The HIV reverse transcriptase routinely isolated from virions is a mixture of the full-length p66 peptide and the p66 peptide truncated at the C-terminal end to yield a p51 peptide. The ratio of these two peptides in the native wild type enzyme is considered to be 1 to 1 (p66 to p51).

In in vitro protein-protein binding experiments, we find that p66 RT binds to our recombinant p51 peptide, representing a C-terminal truncation of the p66 peptide (collaboration with M. Lewis), forming a 1 to 1 heterodimer. The p66 peptide also binds to itself, forming a 1 to 1 homodimer. C-terminal truncation of p51, yielding a p29 polypeptide, completely eliminates complex formation with p66, indicating that the central region of RT may be a dimerization domain responsible for protein-protein interaction. The p15 peptide representing the C-terminal end of p66 is unable to bind to p66.

2. Template·Primer recognition by purified HIV reverse transcriptase was further investigated. Earlier kinetic studies indicated that the reaction pathway for DNA synthesis is ordered, with template-primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988). A pre-steady state kinetic approach was employed to obtain binding constants for the first step in the DNA polymerization reaction scheme, enzyme and template·primer binding. The p66 homodimer and the p66:p51 heterodimer forms of the RT were compared. The K_D values for template·primer [poly r(A) oligo d(T)] binding were similar for the two enzymes and were close to values obtained earlier by other groups (Richardson, Eckstein and Goody). The enzyme-template·primer complex dissociation rate, k_{off} , was complex for each enzyme, showing a rapid-dissociation component and a slow-dissociation component. Measurements of the stoichiometry of enzyme plus template·primer binding indicated that the RT dimer molecule binds to a single template·primer, rather than two or more template·primers. Since both subunits of the RT dimer are capable of binding primer, as purified monomers, these results indicate that one of the two binding pockets in each dimer RT is squelched.

We found that enzyme primer complex formation can also be examined using UV cross-linking. Complexes between the p66 homodimer form of reverse transcriptase and the primer analogues oligo d(T)₈ and oligo d(T)₁₆ are detected in high yield using UV cross-linking. Cross-linking, therefore, is seen as a way of measuring the amount of enzyme-primer complex formed under equilibrium binding conditions. We have used this approach to determine the salt sensitivity of the primer binding reaction (i.e., number of ionic interactions) and selected thermodynamic properties of binding. Further, using competition assays with labeled oligo d(T)₈ as probe, we have determined a binding constant for the natural RNA primer (lysine tRNA₃); the enzyme binds very tightly to its natural primer (collaboration with D. Hatfield, NIH, and R. Suhadolnik, Temple University).

Finally, the location of the primer binding site on the surface of the reverse transcriptase is being analyzed using the UV cross-linking approach. The cross-linked enzyme, containing labeled primer probe, has been digested with V8 protease, and peptides have been resolved by SDS-PAGE. Peptides containing label were recovered in good yield and sequenced in this laboratory (assistance of C. Klee). The cross-linking site for oligo d(T)₁₆ was found to be in the N-terminal one-half of p66.

3. The clinically important drugs AZT and ddI exhibit a slight degree of toxicity toward normal cells, and one of the most likely targets for this effect are the cellular DNA repair enzymes DNA polymerases alpha and beta. We have overproduced active human DNA polymerase beta from E. coli and now are collaborating with other laboratories to use this normal cellular enzyme in parallel with HIV RT during in vitro drug screening and drug design studies.
4. DNA-dependent DNA synthesis in vitro by HIV-1 reverse transcriptase (RT) is relatively error-prone. The enzyme, whether recombinant or from virus particles, produces errors while replicating M13mp2 DNA at a rate that, if operative in vivo, would produce about five mutations per genome per round of replication. Sequence analysis of mutants resulting from in vitro synthesis demonstrates that the enzyme has unusual error specificity. Base substitution and one base frame shift mutational hot-spots are observed. The specificity and position of errors suggest that some of the differences (in sequence) observed among HIV isolates could be due to the kinds of errors we are studying in vitro. We continued our analysis of mechanisms of the frameshift and single-base substitution mutations by HIV-1 RT during replication of a heteropolymer DNA template (collaboration with T. Kunkel). We had found earlier that most of the mutations produced by this so-called "error prone" DNA polymerase could be explained by a template-primer misalignment which occurred at sites along the template where the enzyme fall off and must reinitiate synthesis in order to fully replicate the template. In the past year we produced two mutated templates with strategically designed base substitutions that were found to alter this fall off-reinitiation pattern. We found also that mutations during replication of the mutant templates were different: Base alterations that lead to enzyme processivity at a certain template site eliminated plus-one frameshift mutations at this site. Thus, primer misalignment is an important mechanism for mutations of the HIV-1 RT in vitro.
5. Cassette mutagenesis of the RT was undertaken to produce a large series of Alanine single-residue substitution mutants in a region of the enzyme (residues 250 to 271) corresponding to a portion of the putative catalytic center. Arginine substitution also was made for two of the positions, S252 and W253. Highly purified preparations of these modified enzymes were studied for DNA polymerase activity and RNase H activity. Most of the Alanine mutants were similar to wild type in activity, including a mutant at residue K 263 which had been identified as an active site PLP target by the Modak group. The two Arginine mutants, on the other hand, failed to conduct either polymerase or RNase H activity. The Arginine (W253R) mutant at W253 also was studied for

primer binding capacity and was found to be devoid of binding. The mutant enzymes are under study, but the preliminary results to date suggest 1) that a knockout of primer binding capacity blocks both polymerase and RNase H and 2) that K263 is not essential for these activities.

We made use of proteolytic digestion with trypsin to conduct domain mapping of the ~66,000-M_r RT polypeptide. A ~29,000-M_r fragment from the N-terminal portion of RT was found to be relatively trypsin resistant and was produced in sufficient quantity for characterization. This fragment was capable of strong primer binding activity, as revealed by the technique of Southwestern blotting using ³²P-labeled oligo d(T) as probe. The fragment also is able to bind the natural primer for viral replication, lysine tRNA³. These results with the ~29,000-M_r trypsin peptide are consistent with the results described above on localization of the oligo d(T) cross-linking site to the N-terminal portion of RT or the C-terminal end of the 29,000-M_r trypsin peptide.

Publications:

Becerra P, Clore GM, Gronenborn AM, Karlstrom AR, Stahl SJ, Wilson SH, Wingfield PT. Purification and characterization of the RNase H domain of HIV-1 reverse transcriptase expressed in recombinant *E. coli*, FEBS Lett 1990;270:76-80.

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Becerra PS, Kumar A, Lewis M, Widen SG, Karawya E, Abbotts J, Shiloach J, Wilson SH. Protein-protein interactions of HIV-1 reverse transcriptase, Biochemistry, in press.

Abbotts J, Wilson SH. Mechanistic analysis of HIV-1 reverse transcriptase (Review). In: Kumar A ed. Advances in molecular biology and targeted treatment for AIDS. New York: Plenum Press, in press.

Abbotts J, Kumar A, Becerra P, Wilson SH. Inhibitors of HIV-1 reverse transcriptase and mechanisms of mutations during in vitro DNA replication, (Review). J Enzyme Inhibition, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08212-17 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
M. S. Krug	Guest Researcher	LB	NCI
A. R. Sburlati	Visiting Associate	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

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INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.75

PROFESSIONAL:

4.75

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors B
- (a2) Interviews Note: 25% of this effort is AIDS-related

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

Prothymosin α is a highly acidic, nuclear protein which is known to accumulate in proliferating cells. Its function has been investigated by means of four different antisense oligodeoxyribonucleotides directed toward different regions of prothymosin α mRNA. In every case, when synchronized human myeloma cells were released from stationary phase by incubation in fresh medium containing antisense oligomers, cell division was prevented or inhibited. The sense counterparts of these oligomers had no effect. A detailed analysis of antisense-treated cells indicated that growth inhibition was reversible; the cells divided one cell division late relative to sense-treated or untreated myeloma cells. The failure to divide correlated directly with a deficit in prothymosin α and with the survival, primarily in the nucleus, of intact antisense oligomers. Our data suggest that prothymosin α is essential for cell division.

The two forms of prothymosin α mRNA were characterized and found to arise by alternative splicing of one functional gene. As a result of nonconsensus splice acceptor selection, a GAG codon, 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 (0.5% of prothymosin α mature mRNA in placenta) contains the GAG codon and encodes an identical protein made one residue longer by the insertion of glutamic acid at position 40 of the protein. This is the first example of alternative splicing in which one gene, without adding, subtracting, or substituting exons, gave rise to two almost identical transcripts, with the larger acquiring an triplet introduced, in frame, into sequences specifying the fully processed protein.

Reverse transcriptase from human immunodeficiency virus uses one DNA:RNA binding site for polymerization of complementary DNA on RNA templates or for hydrolysis of RNA in DNA:RNA hybrids.

Project DescriptionProthymosin α 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 precise 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 clones prothymosin α cDNAs lacked an amino-terminal signal peptide; (2) the protein contained a functional nuclear targeting 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 localized exclusively in the nucleus. Instead, we propose a role in cell proliferation as a result of the following experimental findings: (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; and (3) prothymosin α is induced in normal human lymphocytes and in serum-starved NIH 3T3 cells upon growth stimulation with mitogens or serum, respectively.

In the past year we have directly evaluated the relationship between prothymosin α and the division of a synchronized population of human myeloma cells by making use of antisense oligodeoxyribonucleotides. Release from stationary phase by incubation in fresh medium containing any of four different antisense oligomers directed towards mRNA sequences lying athwart the initiation codon, immediately downstream of the first ATG triplet, near the translation stop codon, or deep in the 3' noncoding region, respectively, resulted in either complete cessation of cell growth or severe growth inhibition. In contrast, treatment with the corresponding sense oligomers or with an antisense oligomer randomized by reversing the sequence from 5' to 3' had no effect on cell division. Growth inhibition measured 24 h after incubation with antisense oligomers occurred regardless of whether the cells were rapidly dividing or recovering from growth arrest. The precise time interval between division of control cells and cells in the process of overcoming antisense-induced

repression of growth was established; antisense-treated cells divided one cell division late. The ability to divide correlated with the amount of prothymosin α found in the cell. Because we have no useful antibodies which recognize prothymosin α , we developed an assay for the protein by using two dimensional gel electrophoresis to establish the amount of [^3H]glutamic acid-labeled prothymosin α accumulated in the cell. We found that antisense-treated cells incurred a deficit in prothymosin α amounting to 75%. By measuring prothymosin α mRNA levels during the same interval, we established that antisense oligomers did not stimulate degradation of mRNA, presumably by creating substrates for endogenous ribonuclease H activities, but instead, caused hybrid arrest of translation. We also found that the time course of antisense-induced repression of growth was consistent with the stability of antisense oligomers inside the cell. Whereas sense oligomers were degraded almost instantaneously, intracellular antisense oligomers persisted for 24 h, predominately in the nucleus. These results depended on an innovative labeling procedure which introduced [^{32}P] only into phosphodiester bonds, and a method for recovering and quantifying intact oligomers, both developed by us. The study shows that prothymosin α is essential for cell division.

In order to quantify prothymosin α in cells, we devised a procedure for purifying the protein to homogeneity in two steps. The technique is based on the ability of prothymosin α , virtually alone among proteins, to enter the aqueous phase of a phenol extraction; separation from cellular RNAs was accomplished by SDS polyacrylamide gel electrophoresis. Using this method, we found that 50 μg of purified prothymosin α can be obtained from 3×10^8 rapidly growing human myeloma cells, or that 0.02% of the total protein in the cell is prothymosin α . We also debunked the notion, propounded by others, that prothymosin α is covalently attached to an RNA. Electrophoretic analysis of natural prothymosin α indicated that it comigrated with synthetic prothymosin α generated in the wheat germ system by translation of a synthetic, capped prothymosin α mRNA. Furthermore, the protein, upon carbohydrate analysis, was found to be devoid of ribose.

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 40. The two transcripts might arise as a result of alleles at the same locus or by alternative splicing of a single gene. We ruled out alleles by discovering that 22 human genes for prothymosin α were identical at the boundary of intron 2 and exon 3 where the extraneous GAG codon occurred. We favor alternative splicing because the sequence in question, $(\text{Py})_6\text{C}(\text{Py})_2\text{GAG}^{\downarrow}\text{GAG}^{\downarrow}$, provided two closely spaced splice acceptor sites, indicated by the vertical arrows. 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 revealed that the smaller prothymosin α transcript prevails, with the larger either undetectable, or, for example, present in placenta at 0.5% of prothymosin α transcripts. Furthermore, neither tissue type nor transformation affects splice site selection. Our data indicate that the common mRNA is made in a splicing event which would be expected to be rare because the first AG dinucleotide following the intronic polypyrimidine tract is not employed. This is the first example of alternative splicing in which one gene, without adding, subtracting, or substituting exons, gives rise to two almost identical transcripts, with the larger acquiring a triplet introduced, in frame, into sequences specifying the fully processed protein. The possibility of creating a protein with altered function by aberrantly splicing the transcript of a normal gene which can also produce the normal product may add a new dimension to the analysis of cellular metabolism.

AIDS Research: Ribonuclease H

Objectives:

Reverse transcriptase has two activities: a polymerase activity which synthesizes double-stranded DNA from genomic RNA; and a ribonuclease H (RNase H) function which degrades genomic RNA after reverse transcription has taken place. We plan to determine the mechanism of the RNase H activity and to distinguish features that are either common to or different from those of the polymerase activity. We also aim to identify and characterize inhibitors.

Major Findings:

Kinetic constants governing the binding of substrates and products to reverse transcriptase have been determined and refined, using the RNase H activity as an assay. These values have been compared with constants governing the polymerase reaction. The inhibition of both enzymatic activities by ribonucleoside vanadyl complexes has been studied, and several derivatives of these complexes have been evaluated in the RNase H reaction. Our findings support the conclusion reached earlier: a single substrate binding site for the DNA:RNA hybrid serves both of the catalytic functions. We have also found that deoxyribonucleoside triphosphates enhance RNase H catalysis by facilitating or stabilizing dimerization of the subunits of recombinant reverse transcriptase derived from human immunodeficiency virus.

Publications:

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SUMMARY STATEMENT

LABORATORY OF MOLECULAR BIOLOGY

DCBDC, NCI

OCTOBER 1, 1990 to SEPTEMBER 31, 1991

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. The Laboratory of Molecular Biology has undergone several major personnel changes during the past year. Dr. Kenneth Yamada has left to become Chief of the Laboratory of Developmental Biology, National Institute of Dental Research, and Dr. Bruce Howard has also left to become Chief of the Laboratory of Molecular Growth Regulation in the National Institute of Child Health and Development. This has created an opportunity to expand some of the existing groups and to make several new appointments. Dr. B. K. Lee will establish a Section of Molecular Modelling and Dr. Sheue-yann Cheng will become Chief of the Section of Gene Regulation.

Immunotoxin and Oncotoxin Therapy of Cancer

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

The goal of this project is to create new cytotoxic agents for the treatment of cancer and other diseases by fusing cell targeting genes to genetically modified forms of *Pseudomonas* exotoxin gene. This is done by fusing DNAs encoding growth factors, single chain antibodies, or other cell recognition molecules to DNAs encoding modified forms of *Pseudomonas* exotoxin. The chimeric toxin molecules are expressed in *E. coli* and purified to homogeneity. Using this approach, we have made TGF α -PE40, IL2-PE40, IL4-PE40, IL6-PE40, CD4-PE40, IGF1-PE40, acidic FGF-PE40 and several single chain antibody toxin fusion proteins including anti-Tac(Fv)-PE40, anti-transferrin(Fv)-PE40 and B3(Fv)-PE40. TGF α -PE40 kills cells with EGF receptors and has now been shown to have an antitumor effect in mice when injected I.P. against both a epidermoid carcinoma and a prostate cancer growing subcutaneously. TGF α -PE40 is being developed by Merck Inc. for the therapy of bladder cancer; clinical trials are planned for late this summer. IL2-PE40 is very effective in killing mouse and rat cells with IL2 receptors but is much less active against primate and human cells. Several modifications have been made to produce a more active reagent. The most active of these is a single chain immunotoxin anti-Tac(Fv)-PE40 which is extremely cytotoxic to human and primate cells containing IL2 receptors including cells directly isolated from patients with adult T cell leukemia. Efforts are being made to scale up the production of this molecule for a clinical trial. This is being done in collaboration with Protein Design Labs. IL6-PE40 and a variant IL6-PE66^{4Glu} are cytotoxic to several myeloma cell lines and hepatoma cell lines; hepatoma cells with as few as 400 receptors per cell can be killed by IL6-PE66^{4Glu} and this material has been shown to have an antitumor effect in mice bearing human hepatomas. Because tumors are dependent on new blood supply, we have constructed an agent employing acidic-FGF to target either PE40 or PE66^{4Glu}. These agents are cytotoxic to FGF receptor bearing cells and are now undergoing preclinical evaluation. We previously reported that CD4-PE40 kills HIV infected cells by binding to the gp120 present on the surface of these cells. We have now found that the combination of AZT and CD4-PE40 is

synergistic and will arrest the spread of HIV infection in cultured cells and sterilize infected cell cultures. CD4-PE40 is being prepared by Upjohn and will enter clinical trials in the summer of 1991. A novel monoclonal antibody (B3) that is reactive with many colon, breast and ovarian tumors has been isolated. In addition, the genes encoding the variable regions of this monoclonal antibody have been cloned and fused to PE40 to create B3(Fv)-PE40. This single chain immunotoxin has been shown to have a striking antitumor effect against human tumors growing in nude mice and is also undergoing preclinical development. Several mutant forms of PE have been synthesized which have increased activity. This has been done by changing the carboxyl terminus of PE40 from REDLK to KDEL. Fusion proteins ending in KDEL are two to ten-fold more cytotoxic to target cells than those containing the native sequence. PE is a very immunogenic molecule. To permit prolonged therapy with immunotoxins, the effect of an immunosuppressive agent, 15-deoxyspergualin, has been studied by treating mice receiving immunotoxins with this drug. 15-deoxyspergualin has been found to completely suppress the primary antibody response to PE in mice. We have previously shown that a 37 kDa fragment of PE containing the ADP ribosylating domain is translocated into the cytosol. We have studied this process by introducing foreign polypeptides into this region of the PE molecule to determine if they can also be translocated. We have inserted barnase, a ribonuclease, close to the end of domain III and shown that it is translocated into the cytosol. We have inserted other peptides within domain Ib and shown these are also translocated into the cytosol. Thus PE can be used to introduce foreign peptides into the cytosol of cells for various purposes including for presentation to the MHC class I system.

Development of Immunotoxins for Cancer

D. FitzGerald

Pseudomonas exotoxin (PE) is cleaved by cells to produce fragments of 28 kD, from the N-terminus, and 37 kD, from the C-terminus. This step is necessary to generate the C-terminal fragment which contains ADP-ribosylating activity and is translocated to the cytosol. D. FitzGerald has shown that mutant forms of PE that cannot be cleaved, are not translocated to the cytosol and are not toxic. The protease responsible for cleavage is a membrane-associated enzyme and its activity can be detected in crude membranes from L929 cells. The pH optimum for cleavage is 5.5. Cells were fractionated on Percoll gradients and the proteolytic activity was found in fractions corresponding to endosomes but not in lysosomes. The cleavage of PE by membranes is stimulated by divalent cations and inhibited by EDTA. However, inhibitors of serine proteases and other inhibitors of metalloproteases do not affect cleavage. Membrane preparations cannot cleave PE276G, a mutant form of PE previously shown to be nontoxic because cells failed to generate a 37 kD fragment. Protease activity can be solubilized using non-ionic detergents (NP-40, Octyl-glucoside). Fractions with proteolytic activity have been recovered after ion exchange and gel filtration chromatography. The site of cleavage within the PE molecule is an arginine-rich loop, close to arginine 279. Mutagenesis of the PE gene close to arginine 279 has located residues that are needed for proteolysis and toxicity. D. FitzGerald has also identified a PE-binding protein, thought to be the PE-receptor. The receptor is present in crude membranes and detergent extracts, its role in the proteolysis step is currently not understood.

Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells

M.C. Willingham

There are many changes in the properties of cells following malignant transformation. M. C. Willingham has concentrated on changes in the expression of surface molecules that might represent tumor-specific markers that could be exploited both for diagnosis and therapy of human tumors. Using hybridoma technology and

morphologic methods, monoclonal antibodies to surface epitopes on human tumor cells were isolated. One of these antibodies, B3, has recently been studied in detail. This antibody has been shown to react with a specific area of the Lewis^y antigen structure. This antibody reacts with some normal tissues, such as gastric and bladder epithelium, as well as human tumors of colon, ovarian and breast origin. A conjugate of B3 with *Pseudomonas* exotoxin (PE) has been shown to be effective in killing target tumor cells in tissue culture, as well as in an animal model in nude mice using both A431 and MCF-7 human tumor cells. The absence of serious tissue-specific toxicity in preclinical testing in monkeys suggests that B3 may be useful for therapy of some human cancers. Another mAb, K1, reactive with ovarian tumors and normal mesothelium has been isolated and characterized. It reacts with an antigen that is co-expressed frequently with the CA125 antigen, but it does not react with purified CA125. The antigen reactive with K1 (CAK1) is not shed from the surface of cells, yet it is released from cells treated with phospholipase C. CAK1 has been partially purified and appears to be a protein of ~40 kD. In other studies, a morphologic assay for the monomer-tetramer conversion of pyruvate kinase M1 in intact cells was developed; this enzyme is a major binding protein for thyroid hormone in the cytoplasm of cells. Also, morphological analysis of a transgenic mouse line expressing the human EGF receptor gene in Metestis showed that these mice have a defect in microtubule stability in the axoneme of sperm.

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells

I. Pastan and M.M. Gottesman

The simultaneous resistance of cancer cells to many different anti-cancer drugs is the major impediment to successful chemotherapy of metastatic disease. An important mechanism of multidrug resistance is expression of P-glycoprotein, a 170,000 dalton energy-dependent drug efflux pump which removes natural product drugs from the cell. In order to study how this pump removes drugs from cells, an *in vitro* vesicle system was developed and ATP has been shown to be the preferred energy source. Many of the drugs which are transported compete with each other for a single site or small number of sites on the transporter. Labeling sites for the P-glycoprotein inhibitor ³H-azidopine occur in both the amino and carboxy-terminus of the protein, and these two sites appear likely to make up a single channel through which the drugs move. Molecular manipulations have identified the first intracytoplasmic loop as a domain involved in drug recognition, which is distinct from the drug labeling sites identified with ³H-azidopine. In collaboration with G. Merlino, a *MDR1* transgenic mouse has been developed whose bone marrow is protected from the cytotoxic effects of anti-cancer drugs by expression of P-glycoprotein. This model has been used to identify potent agents which inhibit the multidrug transporter *in vivo*, since these agents sensitize the transgenic mice to the leukopenia induced by chemotherapy. The *MDR1* cDNA can also be introduced into mouse bone marrow by retroviral infection. Such *MDR1* retroviral vectors should be useful for gene therapy to protect bone marrow during cancer therapy and to introduce non-selectable genes into bone marrow. New *in vitro* models of resistance to VP-16 and cis-platinum, not involving the multidrug transporter, are under development.

Regulation of Gene Activity

I. Pastan and A. Johnson

The EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To study regulation of the EGFR gene, the promoter region was isolated and shown to contain many transcription factor binding sites. The expression of the EGFR gene is governed by the interaction with the GC-rich promoter region of the gene of transcription factors that activate (Sp1 and ETF) or repress (GCF) transcription. To examine the

relationship between EGFR and GCF expression, we have analyzed the level of GCF RNA in human tissues and cancer cell lines. We have also examined the level of GCF RNA after treatment of cells with agents that regulate EGFR expression. Three RNA species hybridize to the GCF cDNA and fragments of the cDNA have been used to determine the relationship of these RNA species. The chromosomal localization of the GCF gene has been determined by *in situ* hybridization. The GCF cDNA has been placed under control of the metal inducible metallothionein promoter and three lines of transgenic mice have been generated that express GCF mRNA.

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

G. T. Merlino and I. Pastan

The advent of transgenic technology, in which foreign genetic information is stably introduced into the mammalian germ line, has provided a powerful approach to the study of gene function and regulation. This approach is being used to investigate several important aspects of cancer pathogenesis: the role of growth factors, receptors and oncogenes in the initiation and development of neoplasia, and the ability of malignantly transformed cells to evade destruction by chemotherapeutic agents.

Transforming growth factor alpha (TGF α) and epidermal growth factor (EGF) stimulate cellular proliferation by binding and activating the EGF receptor tyrosine kinase. Perturbation of this signal transduction pathway can transform cells in culture, and has been implicated in the development of human cancer. To examine this hypothesis *in vivo*, transgenic mice were made bearing either the human TGF α or EGF receptor gene. TGF α overexpression induces hepatocellular carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and gastric cystic hyperplasia. Furthermore, analysis of these transgenic mice has led to new discoveries about normal development of the breast and testis. Transgenic mice made using an activated form of a related gene, int-3, which contains EGF repeats and is a member of the Notch gene family, developed hyperplasia of secretory epithelia and neoplasia of the salivary and mammary glands. In addition, male mice were sterile and female mice could not lactate.

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

PROJECT NUMBER

Z01 CB 08000-21 LMB

PERIOD COVERED
 October 1, 1990 to September 31, 1991

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

Regulation of Gene Activity

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

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

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

3.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To study regulation of the EGFR gene, the promoter region was isolated and shown to contain many transcription factor binding sites. The expression of the EGF receptor (EGFR) gene is governed by the interaction with the GC-rich promoter region of the gene of transcription factors that activate (Sp1 and ETF) or repress (GCF) transcription. To examine the relationship between EGFR expression and GCF expression, we have analyzed the level of GCF RNA in human tissues and cancer cell lines. We have also examined the level of GCF RNA after treatment of cells with agents that regulate EGFR expression. Three RNA species hybridize to the GCF cDNA and fragments of the cDNA have been used to determine the relationship of these RNA species. The chromosomal localization of the GCF gene has been determined by *in situ* hybridization. The GCF cDNA has been placed under control of the metal inducible metallothionein promoter and three lines of transgenic mice have been generated that express GCF mRNA.

Major Findings

The EGFR promoter is very GC rich and binds a number of transcription factors that are specific for GC rich sequences. A new transcription factor, GCF, has been shown to bind to three sites in the EGFR promoter and to repress transcription. We have now examined the expression of GCF mRNA in cancer cell lines. HUT-102 cells, derived from a T-cell lymphoma, expressed the highest level of GCF mRNA. Two gastric cell lines, AGS and KATO III, also express elevated GCF mRNA levels. High levels were also noted in FEM-X (melanoma) and U266 B1 (myeloma) cell RNA. No GCF RNA was detected in WI38 fibroblasts. GCF mRNA was present in all human tissues examined with the highest level found in esophagus.

The GCF cDNA probe was shown to hybridize to three RNA species (4.5, 3.0 and 1.2 kb). The 4.5 kb RNA species hybridized to the 5' region of the GCF cDNA (1-561), whereas, the 3.0 and 1.2 kb RNA species hybridized to all regions of the GCF cDNA. The GCF cDNA was used to determine the chromosomal localization of GCF which is located at 2 p11.1-11.2.

GCF mRNA was not induced by EGF or serum but was elevated 5-fold by PMA. The kinetics of GCF mRNA induction by PMA had an inverse correlation with EGFR mRNA induction. EGFR mRNA began to increase only after GCF mRNA levels began to decrease.

We have generated three lines of transgenic mice that express GCF mRNA. The GCF mRNA is under control of the metal inducible metallothionein promoter and has the human growth hormone polyadenylation signal. The F₁ offspring of founder mice exhibit no phenotypic changes to date. Homozygous lines are being generated and tissue RNAs from the mice are being examined for levels of GCF RNA, EGFR RNA and β actin RNA.

Publications:

None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08010-18 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells

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

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

Other: I. Pastan Chief, Laboratory of Molecular Biology NCI
 K. Chang Visiting Associate LMB, NCI
 G. Merlino Expert LMB, NCI
 S.-y. Cheng Chemist LMB, NCI
 A. Rutherford Biologist LMB, NCI

COOPERATING UNITS (if any)

Laboratory of Cell Biology, DCBDC, NCI, NIH

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

3.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

There are many changes in the properties of cells following malignant transformation. We have concentrated on changes in the expression of surface molecules that might represent tumor-specific markers that could be exploited both for diagnosis and therapy of human tumors. Using hybridoma technology and morphologic methods, we have isolated monoclonal antibodies to surface epitopes on human tumor cells. One of these antibodies, B3, has recently been studied in detail. This antibody has been shown to react with a specific area of the Lewis^y antigen structure. This antibody reacts with some normal tissues, such as gastric and bladder epithelium, as well as human tumors of colon, ovarian and breast origin. Recently, a conjugate of B3 with Pseudomonas exotoxin (PE) has been shown to be effective in killing target tumor cells in tissue culture, as well as in an animal model in nude mice using both A431 and MCF-7 human tumor cells. The absence of serious tissue-specific toxicity in preclinical testing in monkeys suggest that B3 may be useful for therapy of some human cancers. Another antibody, K1, reactive with ovarian tumors and normal mesothelium has been characterized. It reacts with an antigen that is co-expressed frequently with the CA125 antigen, but it does not react with purified CA125. The antigen reactive with K1 (CAK1) is not shed from the surface of cells, yet it is released from cells treated with phospholipase C. CAK1 has been partially purified and appears to be a protein of ~40 kD. An immunotoxin prepared using K1 is effective in killing target cells in tissue culture. In other studies, a morphologic assay for the monomer-tetramer conversion of pyruvate kinase M1 in intact cells was developed; this enzyme is a major binding protein for thyroid hormone in the cytoplasm of cells. Also, other studies of a transgenic mouse line developed using the human EGF receptor gene showed that these mice have a defect in microtubule stability in the axoneme of sperm in homozygous animals.

Major Findings:

As part of our program of isolation of monoclonal antibodies to tumor-related antigens on the surface of human tumor cells, we have characterized the properties of B3, an antibody reactive with tumors from colon, ovary and breast. Characterization of the antigen reactive with B3 has shown that this antibody recognizes a portion of the Lewis^y antigen carbohydrate structure. This antibody reacts with some normal epithelia, such as in stomach and bladder, in both human and monkey normal tissues. An immunotoxin prepared with B3 has been shown to be effective in tumor cell killing in tissue culture, as well as in nude mice bearing tumors from the A431 and MCF-7 human tumor cell lines. Preclinical toxicity testing of a B3 immunotoxin in monkey has shown that there was no significant targeted toxicity in the normal tissue sites shown to react with B3 by immunohistochemistry. This suggests that B3 may be useful in the therapy of some human cancers.

Another antibody isolated during this project was K1. This antibody recognizes an antigen (CAK1) that is frequently co-expressed with the CA125 antigen commonly found in non-mucinous tumors of the ovary. However, K1 does not react with purified CA125, and is not shed into the medium of culture cells bearing this surface antigen, or into the blood of patients with ovarian cancer, as seen with the CA125 antigen. CAK1 is found in normal mesothelium in both humans and monkeys, as well as in amniotic membrane in placenta. It is also found in some human tumors other than ovarian tumors, such as squamous tumors of cervix and esophagus. Biochemical characterization of CAK1 has shown that the antigen appears to be a ~40 kD protein that is released from the cell surface after treatment with phospholipase C. We have developed a competition assay for the presence of this antigen using iodinated K1 antibody, and have begun attempts to clone the gene coding for the CAK1 protein. In other studies, this antigen was found to be expressed frequently in epithelioid mesotheliomas. An immunotoxin prepared using K1 has shown targeted cell killing in tissue culture. Since this antibody is not rapidly internalized by cells, it may be useful in combination with radionuclides for therapy and in imaging of tumors.

In other studies, in collaboration with G. Merlino, we have characterized the histochemical and morphological features of a transgenic mouse line generated by introduction of the human EGF receptor. This line of mice shows infertility of the male homozygotes. Microscopic examination of these mice showed expression of the transgene selectively during spermiogenesis. These homozygous mice had immotile sperm with unstable axonemes which lost about one-half of their doublet microtubule pairs during transit of the early portion of the epididymis. Since this defect was seen only in homozygous male mice, this probably represents the knock-out of a normal gene necessary for spermiogenesis and microtubule stability.

In other studies, in collaboration with S.-y. Cheng, we have used a monoclonal antibody (J13) that selectively recognizes the monomeric form of pyruvate kinase (PKM), an enzyme of central importance in glycolysis and energy metabolism. PKM is the major binding protein in the cytoplasm of cells for thyroid hormone (T3). The enzymatic activity of this enzyme is regulated by its polymerization state, with the tetrameric form being very active, and the monomer being inactive. In the test tube, this enzyme's polymerization state can be regulated directly by thyroid hormone. Antibody J13 reacts with the monomeric form of PKM, but does not react with the tetrameric form of PKM in solution. We examined the reactivity of the J13 antibody with intact fixed cells, and were able to show that the immunofluorescence localization with this antibody changed in relation to the cell's PKM monomer/tetramer ratio, a property that can be altered by manipulating the

extracellular glucose concentration. Thus, we can examine the state of this enzyme in intact cells without biochemical purification. These studies may allow a clearer understanding of the ability of thyroid hormone to regulate cell metabolism.

Publications:

Kozak RW, Lorberboum-Galski H, Jones L, Puri RK, Willingham MC, Malek T, FitzGerald DJ, Waldmann TA, Pastan I. IL-2-PE40 prevents the development of tumors in mice injected with IL-2 receptor expressing EL4 transfectant tumor cells, *J Immunol* 1990;145:2766-71.

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Cenciarelli C, Currier SJ, Willingham MC, Thiebaut F, Germann UA, Rutherford AV, Gottesman MM, Barca S, Tombesi M, Morrone S, Santoni A, Mariani 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 multidrug-resistant KB and CEM cell variants. *Int J Cancer* 1991;47:(in press).

Pastan I, Willingham MC, Gottesman MM. Molecular manipulations of the multidrug transporter: a new role for transgenic mice, *FASEB J.* 1991, (in press).

Handler JS, Horio M, Willingham M, Pastan I, Gottesman MM. Polar localization of the multidrug transporter in epithelia results in transepithelial transport of substrates. *ICS877*(book), Elsevier Science Publishers, Amsterdam, 1991, (in press).

Gottesman MM, Horio M, Handler J, Raviv Y, Galski H, Mickisch G, Merlino G, Willingham MC, Pastan I. Function of the multidrug transporter. In: *Drug Resistance as a Biochemical Target for Cancer Chemotherapy.* Orlando, FL: Academic Press, 1991, (in press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08710-14 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

DNA Replication *in vitro*

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

PI: S. Wickner

Research Chemist

LMB, NCI

COOPERATING UNITS (if any)

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

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TOTAL MAN-YEARS

1.0

PROFESSIONAL:

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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.)

I have been interested in the initiation of DNA replication and the regulation of replication and have been using an *in vitro* replication system that replicates DNA carrying the plasmid P1 origin of replication. This reaction requires the P1 RepA initiator protein, which binds to five sites in the P1 origin, and many host proteins including, DnaA, DnaB, DnaC, DnaG, RNA polymerase, DNA gyrase and DNA polymerase III. I have found that three of the *E. coli* heat shock proteins, DnaJ, DnaK (the hsp70 homolog) and GrpE are also required. In collaboration with J. Hoskins and K. McKenney, we have found that DnaJ and DnaK, in an ATP dependent reaction, stimulate the P1 origin specific DNA binding activity of RepA by about 100-fold. We have shown that the mechanism of activation is the conversion of RepA dimers into monomers and that the monomer form binds with high affinity to P1 origin DNA. Reversible chemical denaturants also convert RepA dimers to monomers and simultaneously activate P1 origin DNA binding. Increasing protein concentration converts monomers to dimers and deactivates RepA.

We have also been studying the regulation of P1 DNA replication. The plasmid control region contains nine repeats of the RepA binding site. However, the simple explanation of regulation by titration of RepA has been ruled out by *in vivo* experiments and our observation that DNA containing the origin and the control locus is not replicated *in vitro* even when RepA is supplied in excess relative to binding sites. RepA can bind simultaneously to the origin and the control region causing the DNA to loop *in vitro*, suggesting that DNA pairing may regulate DNA replication. By rearranging the orientation and the spacing of the RepA sites in the control region and by characterizing the mutant RepA proteins, we hope to understand the mechanism of regulation of replication.

Major Findings:

I have been interested in the initiation of DNA replication and the regulation of replication of plasmid mini-P1. The mini-P1 replicon consists of an origin of replication, a replicon specific initiator protein, RepA, and a control locus. The origin contains five direct repeats of the RepA binding site. Plasmids containing this origin are maintained at high copy number. The replication control region, located 1 kb from the origin, is required for mini-P1 plasmids to be maintained at one copy per *E. coli* chromosome. This region contains nine repeats of the RepA binding site.

I have been studying the mechanism of action of the heat shock proteins in the initiation of mini-P1 replication. My in vitro studies have shown that oriP1 replication catalyzed by crude protein fractions of *E. coli* requires DnaK, the *E. coli* hsp70 homolog, DnaJ, and GrpE, along with many other host proteins. I previously showed that RepA is a dimer in solution and forms a stable complex with DnaJ, containing a dimer each of RepA and DnaJ. In collaboration with K. McKenney and J. Hoskins, we have found that DnaK, in a reaction dependent on DnaJ and ATP, activates the specific P1 origin DNA binding function of RepA by about 100-fold. RepA was the only protein found bound to the DNA.

We sought to define the change in the activated form of RepA to understand the mechanism of action of DnaJ and DnaK. We were able to release RepA from isolated RepA-DNA complexes by 1 M NaCl and found that it remained in the activated form. By analogy with the known role of DnaK and hsp70s in disaggregation of denatured proteins, it seemed possible that DnaK and DnaJ were catalyzing the dissociation of RepA dimers into monomers and that this was the form needed for origin binding. By gel filtration chromatography, we found that the activated form of RepA was indeed a monomer under conditions where the unactivated form was a dimer. The observation that DnaK and DnaJ and ATP convert RepA dimers into monomers suggested that it might be possible to chemically dissociate RepA dimers to produce activated RepA. We found that urea treatment followed by dialysis activated the RepA DNA binding function and simultaneously converted dimers to monomers. Thus, it is sufficient for DnaJ and DnaK to catalyze only the dissociation of RepA dimers; they do not need to modify RepA. If RepA monomers are simultaneously unfolded, the monomers can refold without DnaJ and DnaK. We also found that increasing protein concentration converts monomers to dimers and deactivates RepA. The reversibility of the monomer and dimer forms of RepA confirms that activation and deactivation need not involve a covalent change in RepA, such as phosphorylation or dephosphorylation, catalyzed by DnaJ and DnaK. Moreover, they imply that the stability of the dimer to dissociation in the absence of hsp's or denaturants is the result of a kinetic block.

Our model of how DnaJ and DnaK might function catalytically is analogous to that proposed by Pelham for the heat shock response, but differs in that it emphasizes the role of hsp's with native proteins in unstressed conditions. We suggest that in normal growth conditions, native proteins are identified as targets by DnaK through the specific recognition of a protein tag. In our case, DnaJ tags RepA for binding by DnaK, perhaps by exposing hydrophobic regions. When DnaK binds to the RepA-DnaJ complex, either RepA or DnaJ acts as an allosteric effector of the DnaK ATPase. The hydrolysis of ATP leads to the release of DnaK from the complex and the dissociation of RepA dimers into monomers. DnaJ is also released because only the dimer form of RepA binds DnaJ. Thus DnaJ and DnaK regulate protein function at the level of protein structure. We are currently trying to identify some of the protein-protein interactions suggested by this model.

We have also been studying the control of mini-P1 replication. Mini-P1 DNA carrying the control region in addition to the origin is not replicated in the *in vitro* replication system even when RepA is added in a ten-fold excess relative to binding sites. Replication is restored when seven or more of the RepA sites in the control region are deleted or when wild-type RepA protein is substituted with RepA isolated from *repA* mutants that have increased copy numbers *in vivo*. By electron microscopy, RepA binds simultaneously to the origin and the control region causing the intervening DNA to loop, suggesting that DNA pairing may regulate DNA replication by inhibiting initiation. We are currently trying to determine if DNA looping between the origin and the control locus completely explains regulation or if other factors are involved.

Publications:

Wickner S, Hoskins J, McKenney K. Deletion analysis of the mini-P1 plasmid origin of replication and the role of the *E. coli* DnaA protein, *J Biol Chem* 1990;262:13163-13167.

Wickner S, Hoskins J, McKenney K. Function of DnaJ and DnaK as chaperones in origin-specific DNA binding by RepA. *Nature* 1991;350:165-167.

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, 1991; in press.

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. *Regulatory mechanisms of DNA replication*. Berlin: Springer-Verlag, 1991; in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08714-14 LMB

PERIOD COVERED

Bacterial Functions Involved in Cell Growth Control

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

October 1, 1990 to Sept. 30, 1991

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

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	V. de Crecy Lagard	Visiting Fellow	LMB, NCI
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A. Lobner-Olesen, The Technical University of Denmark

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B

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

We have been studying the role that protein degradation plays in regulating gene expression and have initiated a study on the linkages between chromosome synthesis and partition of chromosomes during cell division. We have continued our study of the mechanism of regulation of capsule synthesis, as a model for understanding the role of unstable proteins as regulators. RcsA, a target for the Lon protease, was shown to interact with another regulator, RcsB, in a temperature sensitive manner. Studies on *rcaA* expression have demonstrated a trans-acting role for a site downstream of the structural gene in regulating *rcaA*. We have continued studies on a new proteolytic activity capable of degrading the Lon substrates, RcsA and SulA, which appears when a multicopy plasmid carrying a gene we have called *alp* is introduced into cells. The new activity is dependent on excision of a cryptic prophage and consequent inactivation of a chromosomal gene encoding a stable RNA. A third energy-dependent protease under study is the two component Clp protease. We are continuing mutagenesis studies of the ClpP subunit to determine the specificity and role of ClpP-dependent ClpP processing for assembly and proteolytic activity. In a separate project, camphor resistant mutations in four different loci have been shown by flow cytometry to lead to increased ploidy. In combination with previous genetic analysis, this suggests these mutations (called *mbr*) define three different coupling events for chromosome number and cell cycle.

Major findings:1. 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 of the positive regulator RcsB. 2) The accumulation of the unstable positive regulator RcsA. 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.

In collaboration with C. Schnaitman at Arizona State University, we have shown that increased capsule synthesis in mutants which are unable to synthesize lipopolysaccharide is blocked in *rscC* (sensor) mutants. Thus this effect is mediated via the first of the two activation pathways, and suggests that RcsC is sensing the absence of polysaccharide on the cell surface, either directly or indirectly.

Mutations in the pleiotropic regulatory loci *cya* (cAMP synthesis), IHF (integration host factor), and *bgly* (HNS, another histone-like protein of *E. coli*) all increase capsule synthesis, but are independent of the RcsC sensor, and therefore may act via the second activation pathway. The basis for their action is being further investigated.

Cells carrying single copies of an *rscA-lacZ* transcription fusion carrying 1 kb of DNA upstream of the *rscA* 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 *rscA*. No obvious open reading frame is encoded by this downstream region; we are investigating the basis for its effect.

We have continued the delineation of the *cps* promoter. A 2.0 kb fragment which confers RcsB and RcsA dependent expression in a *lac* fusion plasmid has been defined and *lac* fusions to subclones of this are being constructed. Sequence has also been obtained for a .6 kb subclone which competes with a functional *cps* promoter. Attempts to detect specific protein binding to these fragments by partially purified RcsA and RcsB have thus far been unsuccessful.

Capsule synthesis in *E. coli* K12 is under temperature regulation. In *lon* cells, which express capsule at high levels at 30°C, capsule synthesis is reduced 100-fold at 37° and above. We have demonstrated that the decreased synthesis at high temperatures in *lon* cells is not due to either new *lon*-independent degradation of RcsA, or the action of the RcsC regulator. We postulated, on the basis of genetic evidence, that the RcsA-RcsB interaction might be temperature sensitive. This prediction is supported by the finding that RcsA* mutant protein, which has an increased interaction with RcsB, stimulates temperature resistant capsule synthesis when the RcsA* protein is protected from degradation (in a *lon*- host).

2. 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.

In collaboration with M. Maurizi (LCB/NCI), we have been generating mutations at the cleavage site of *clpP*, in an attempt to answer two questions: what does Clp recognize about this site, and 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*, and that degradation then proceeds towards the final maturation site.

We are initiating an in depth study of both the regulation of *clpA* synthesis and its *in vivo* function. Strains carrying derivatives of *clpA* under the control of the regulatable *mac* promoter are being constructed, and will be used to identify genes essential only in the absence or presence of Clp. Studies on the expression of a *clpA-lacZ* fusion suggest that expression of *clpA* is tightly linked to the availability of certain amino acids and the state of amino acid control.

3. 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 we had supposed to encode the protease.

The sequence of *slp* suggests that it encodes a site-specific recombinase with homology to the integrase of the defective phage P4. In addition, we found that *alp* also shared homology to a previously undefined P4 gene, designated ORF88. The arrangement of the genes in the chromosome and comparisons to the P4 map suggest that *E. coli* K12 contains a previously undetected cryptic prophage of the P4 family, and that ORF88 is likely to act as a positive regulator for *int* synthesis on induction of this prophage. The cryptic prophage is excised from the chromosome and lost from cells after *alp* expression from the multi-copy plasmid. Janine Trempy, who initiated the project, has continued to collaborate on the sequencing of the cryptic 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 or 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 are now attempting to define the role of the RNA and identify the genes for the induced protease.

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. Clones carrying the mutations are being identified as a preliminary to sequence analysis to define the basis for the mutations.

Publications:

Stout V, Torres-Cabassa A, Gutnick D, Maurizi M, Gottesman S. RcsA, an unstable positive regulator of capsular polysaccharide synthesis, J Bact 1991;173:1738-41.

Trun NJ, Gottesman S. On the bacterial cell cycle: *Escherichia coli* mutants with altered ploidy, Genes & Dev 1990;4:2036-47.

Trun NJ, Gottesman S. Characterization of *Escherichia coli* mutants with altered ploidy, Res Microbiol 1991; in press.

Stout V, Gottesman S. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12, Mol Microbiol 1991; in press.

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

PROJECT NUMBER

Z01 CB 08750-11 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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	S. Adhya	Chief, DGS	LMB, NCI
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	G. Storz	Guest Researcher	LMB, NCI

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TOTAL MAN-YEARS:

3.5

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3.5

OTHER:

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 (a1) Minors
 (a2) Interviews

B

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

CRP: The cyclic AMP receptor protein, CRP, undergoes an allosteric change by binding to cAMP. Only the cAMP•CRP complex can bind to specific DNA sequences near many promoters and activate transcription. Isolation and genetic characterization of *crp* mutants that make either allosterically defective or cAMP-independent CRPs and biochemical studies of the purified proteins have established that: (1) cAMP binding induces amino acid 138 at the hinge region to interact with amino acid 141 for hinge reorientation. A polar-polar interaction between the two sites makes the protein cAMP independent, whereas a hydrophobic amino acid at 138, e.g., 138A or 138V makes the protein allosteric defective. An intragenic suppressor can restore the normal activity of 138A or 138V mutation. The results are consistent with the model that protrusion of the DNA-recognizing F-helix is needed for its DNA major groove interaction. We have crystallized some of these mutants for X-ray diffraction. (2) *In vitro* transcriptions with purified proteins and supercoiled mini DNA circles as template for cAMP dependent transcription of *lac* promoter have shown that one can introduce discontinuity in one of the two strands between CRP and RNA polymerase binding sites and still retain CRP-mediated transcription, suggesting that CRP is unlikely to act by supercoiling DNA locally. (3) In order to test that CRP contacts RNA polymerase for transcription, the CRP binding site was put *in trans* to the promoter by concatenation of two DNA circles; under these conditions CRP did not activate transcription, but did inhibit the basal level of CRP independent transcription, suggesting an interaction, albeit improper, could occur *in trans*.

OxyR: The gene regulatory protein, OxyR, binds to DNA and represses transcription, whereas an oxidized form of OxyR, induced by reactive oxygen species, binds to DNA to activate gene transcription. Whether recognized by reduced or oxidized form of OxyR, the interacting DNA sites unexpectedly show very little sequence similarity. Oxidation of OxyR brings about a conformational change in the protein, which can be reversed by reducing agents. The reversibility is now being studied by mutational changes. The basis of specific DNA recognition is being studied by isolating and characterizing mutant DNA sites.

Major Findings:

We are studying two gene regulatory DNA binding proteins of *Escherichia coli*: CRP and OxyR. Both proteins can repress or activate transcription, but most likely each uses a different mechanism to accomplish repression and activation. For repression and activation, CRP must bind to specific sites on the DNA. In the case of repression, CRP binding simply blocks RNA polymerase binding. For operons where CRP activates, CRP binds to a site located upstream or close to the promoter and activates. CRP can function as a repressor or activator only when bound to cAMP which acts as a signal. OxyR differs from this scenario. OxyR can bind to specific sites on the DNA where it can act as an activator or repressor. Although OxyR binding is sufficient for OxyR mediated repression, only OxyR that has been activated by exposure to oxidizing agents can stimulate transcription. Hence the DNA-bound OxyR is not an activator unless the protein is signaled by oxidizing conditions. Study of these two proteins that can function as transcriptional activators provides opportunities for the study of structural changes in proteins, protein-DNA interactions, protein-protein interactions, and protein conformational changes that are needed for gene regulation. The following studies of CRP and OxyR were successfully performed during 1990-91.

A. The cAMP-induced allosteric change.

Cyclic AMP binding to CRP causes the protein to undergo a conformational change, which is a requirement for site-specific DNA binding. We have further characterized previously reported and newly isolated several classes of CRP mutants that have allowed us to define partly how cAMP causes the allosteric change. Key features of our findings are: (1) cAMP induces a reorientation of the hinge region that connects the cAMP binding domain to the DNA binding domain; and (2) Protrusion of the DNA recognition α -helix from the neighborhood.

1. Mutational changes of amino acid 138 in the hinge from aspartic acid to valine or alanine makes an allosteric-defective CRP, i.e., it binds cAMP normally but does not undergo the conformational change. Proteolytic studies suggest that these mutants, even in the presence of cAMP, are frozen in the conformation that CRP normally has in the absence of cAMP. We have purified these proteins and in a collaboration with Dr. I. Weber, have obtained crystals of them. This is significant because, despite many attempts, no one has been able to crystallize wild type CRP in the absence of cAMP. Once the in-progress diffraction analyses are complete, we hope to have an idea of what the structure of CRP is like before cAMP binds.
2. Taking a genetic approach to study these allosteric-defective mutants, we have isolated intragenic suppressors of the mutant phenotype, that is, mutants that retain the original mutation, yet have another mutation within CRP that allows them to function normally in the presence of cAMP. Several of these mutations have been sequenced. An interesting mutation of this class occurs in an amino acid that would cause pushing of the DNA-binding F α -helix away from the rest of the protein. We had predicted previously, based on other mutants, that pushing away the F α -helix was a critical part of the cAMP-induced conformational change. We are currently studying the protein biochemically.
3. CRP very likely can induce some change in the DNA it binds. Since a CRP dimer can accommodate a spacer of 6-9 bp between the two dyad symmetry elements, TGTGA and TCACA, during DNA binding, we believe that CRP binding induces a change in the helix pitch of the spacer region to maintain a constant distance between the symmetry elements.

B. How CRP activates transcription.

1. CRP may activate transcription by altering DNA conformation in the promoter and/or by contact with RNA polymerase. To investigate these possibilities, we introduced a single-stranded nick between the CRP binding site and the *lac* promoter. We found that CRP-dependent *lac* transcription is normal from the nicked DNA, showing that CRP does not act by locally supercoiling the DNA.
2. The companion experiment to the above is testing whether CRP can act *in trans* by protein-protein interaction with RNA polymerase. In this experiment, the CRP binding site and the promoter were on different pieces of DNA that were concatenated. We found that CRP did not activate transcription when present *in trans*, but could actively inhibit the basal level transcription, suggesting an interaction, albeit improper, could occur *in trans*. We are pursuing this study further.
3. From the crystal structure of the CRP-cAMP complex, we know that the CRP molecule is an asymmetric dimer of identical subunits. The CRP dimer binds to sites that exhibit a hyphenated dyad symmetry, and CRP functions best with one cAMP bound per dimer. To test whether there was a directionality to the way CRP had to bind relative to the *lac* promoter, we created situations where the native CRP binding site was inverted and where the native site was replaced with a site with perfect dyad symmetry. We found there was no difference among the three constructs in activation ability, although the site with perfect dyad symmetry binds CRP 500 times as tightly. We conclude there is no site-generated directionality of DNA-bound CRP with respect to the promoter.

C. Oxidation-reduction of OxyR and DNA binding.

OxyR is homologous to the "LysR" family of bacterial regulatory proteins. The OxyR protein binds to various promoters of *oxyR*-regulated genes, but unexpectedly, these DNA sites show very little sequence similarity. The mechanism of OxyR regulated transcription was elucidated in defined *in vitro* transcription assays. Both the oxidized and the reduced forms of the OxyR protein bind specifically to DNA, and the interactions of the two forms of OxyR with DNA are different, suggesting that reduction and oxidation of the OxyR protein bring about conformational changes that lead to gene repression and activation, respectively. These findings suggest:

1. The OxyR protein must contain a redox-active center which can be oxidized in a specific and reversible fashion; inter- or intra-molecular disulfide bonds meet this criteria. A cofactor or metal associated with OxyR might also be sensitive to oxidation. Alternatively, cysteine or methionine residues might be reversibly oxidized. We are distinguishing between these possibilities by characterizing cysteine and methionine mutants of OxyR as well as examining physical properties of the OxyR protein.
2. The differences between the two forms of OxyR, oxidized-active and reduced-repressive, are being characterized by studying these and mutant forms of the OxyR which are locked in one of the two states. To this end, we are collaborating with Dr. John Kurigan of Rockefeller University. He has been able to grow crystals of the oxidized form of OxyR and is currently doing X-ray analysis.
3. Almost all DNA-binding proteins characterized to date have been found to bind to a defined sequence. Since the OxyR protein binds to sequences that do not show obvious similarity, we are characterizing the basis for OxyR-DNA recognition by characterizing additional natural and mutant binding sites.

Publications:

Storz G, Tartaglia LA, Farr SB, Ames BN. Bacterial defenses against oxidative stress. Trends Genet 1990;6:363-8.

Adhya S. Regulation of gene activity in prokaryotes. In: Essentials of Molecular Biology. Boston, Massachusetts: Jones and Bartlett, 1991, in press.

Zwieb C, Adhya S. Detection of bending of DNA by gel electrophoresis: Use of plasmid vectors. In: Jost JP, Saluz HP. eds. A Laboratory Guide for *in vitro* Studies of DNA-Protein Interactions. Basal, Switzerland: Friedrich Miescher, 1991, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08751-11 LMB
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PERIOD COVERED
October 1, 1990 to September 31, 1991

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)

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<input type="checkbox"/> (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 is negatively regulated by two repressors, GalR and newly discovered GalS. The repression by GalR is strong, whereas that of GalS is moderate, although both act by binding to the same two spatially separated operators, O_E and O_I . Additionally, GalR is epistatic to GalS; GalS-mediated repression is seen only when the cell is genetically devoid of GalR. The gene encoding GalS has been defined by isolating mutations in the *galS* gene and cloning the wild type and mutant genes. The amino acid sequence of GalS, as deduced from DNA sequence, is 85% similar to that of GalR. *galR* and *galS* themselves carry two operators, O_E and O_I , suggesting autoregulation by GalR and/or GalS through DNA looping -- the same way looping operates in the *gal* operon itself.

The biochemical mechanism of negative control of the *gal* operon by GalR repressor has been studied in detail. We have proposed that repression involves "caging" of RNA polymerase by a DNA loop formed by interaction of O_E and O_I bound repressors facilitating a transcription-inhibitory physical contact(s) between repressor and RNA polymerase. Consistent with the model, we have shown that (i) the specificity of the contact between operator-bound repressors is independent of the nature of DNA-protein interactions and resides in the carboxy domain of the repressor protein; (ii) the proposed repressor-RNA polymerase contact may be through the α -subunit of the holoenzyme; and (iii) transcription from both *gal* promoters in a unitary promoter plasmid minicircle is repressed in a purified system with wild type repressor. Repression was not observed with a mutant repressor that binds to the operators but does not loop the DNA.

Major Findings:Negative control by GalR repressor:

The two promoters, $P1$ and $P2$, of the *gal* operon, which are modulated by CRP in opposite fashions are also negatively regulated by GalR repressor. The negative regulation of the promoters is achieved by binding of GalR to two operators, O_E and O_I . From previous genetic studies, we have proposed that negative control of the *gal* operon is achieved by forming a DNA-multiprotein complex of a higher ordered structure in which (i) RNA polymerase, CRP and two repressor molecules bind to the regulatory DNA segment simultaneously; (ii) the two operator-bound repressors interact forming a DNA loop encompassing the promoter region, in which the RNA polymerase is "caged"; (iii) a direct contact between RNA polymerase and repressor makes the polymerase inactive. The following series of observations support various segments of the model.

1. Although LacI repressor can repress the *gal* operon if the two *gal* operators, O_E^G and O_I^G are replaced by *lac* operators, O_E^L and O_I^L . A heterologous combination, $O_E^L-O_I^G$ or O_E^G and O_I^L , is not repressible when both GalR and LacI are present. This suggests that DNA binding is not sufficient and an interaction between identical DNA bound proteins is also necessary for repression.
2. We have been able to change the DNA recognition specificity of LacI repressor by site-directed mutagenesis of the helix-turn-helix region from *lac* operator recognition specificity to *gal* operator recognition specificity. This altered specificity LacI repressor can completely repress the *gal* operon, i.e. $O_E^G-O_I^G$ operator system. Thus, GalR does not have any special feature to be able to repress *gal* operon that LacI does not.
3. We have shown that a *gal* operon with mixed operator, e.g. $O_E^L-O_I^G$ is repressed in the presence of both wild type LacI repressor and the above LacI repressor with *gal* operator recognition specificity. This suggests that repressor-repressor interaction is separate from DNA binding specificity.
4. We have found that overproduction of the α -subunit of RNA polymerase in cells from a multicopy plasmid causes a partial derepression of the *gal* operon, whereas several deletion mutants of the plasmid encoding α gene (*rpoA*) does not. This is consistent with the idea that normal repression is mediated by contact(s) between repressor and α -subunit(s) of the RNA polymerase. Excess free α in the cell may be titrating out the repressor in the DNA-multiprotein complex, thus freeing the promoter bound RNA polymerase from repression. Attempts are being made to isolate mutants of *rpoA* gene which are defective in the proposed interaction with repressor and thus derepressed for the *gal* operon.
5. We have constructed a unitary promoter plasmid which contains only one promoter of choice in negatively supercoiled DNA minicircles, which are generated from a parental plasmid *in vivo* by the use of phage Lambda integrase system. These DNA circles were used to study the mechanism of *gal* repression *in vitro*. In this system, we have first duplicated the dual control *gal* promoters by CRP. We have then demonstrated that wild type LacI repressor completely represses the *gal* operon carrying $O_E^L-O_I^L$ in a totally purified system. Both $P1$ and $P2$, which are regulated by CRP in an opposite fashion, were repressible in this system, as has been observed *in vivo*.
6. We have also shown that a mutant LacI repressor, which unlike the wild type repressor does not repress *in vivo* and does not show DNA looping by electron microscopy but binds normally to DNA, does not repress *in vitro*.

7. Wild type LacI repressor forms a DNA loop *in vitro* as shown by gel electrophoretic mobility assays as well as by the appearance of DNase hypersensitive sites in foot-printing assays. The mutant LacI repressor fails to show either of the two properties, although as stated above binds to operators normally.

The GalS repressor:

We have discovered a new repressor, called GalS, for the *gal* operon. Its effect is to turn down and not to turn off, the rate of *gal* operon expression. This repression is seen only in the absence of GalR. Thus, GalR is epistatic to GalS action. GalS, like GalR, acts by binding to the O_E^G and O_I^G sequences and is inactivated by Galactose. We are studying the epistasis further.

1. We have isolated mutants defective in the GalS function and also cloned the *galS* gene by marker rescue experiments. DNA sequencing of the gene reveals that the deduced amino acid sequence of GalS is 85% similar to that of GalR. *galS* gene has been cloned into a hyperexpression vector system, and GalS protein is now being purified for *in vitro* studies.

2. We have discovered that *galR* and *galS* genes themselves contain operators, O_E and O_I , O_E near the promoters and O_I within the structural gene, as they are in the *gal* operon. These suggest that both *galR* and *galS* may be autoregulated by GalR and/or GalS involving DNA looping. Autoregulation of *galR* and *galS* is being investigated.

Publications:

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Brenowitz M, Mandal N, Jamison E, Adhya S. DNA-binding properties of a Lac repressor mutant incapable of forming tetramers, *J Biol Chem* 1991;266:1281-8.

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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, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER	
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 CB 08752-11 LMB	
PERIOD COVERED			
October 1, 1990 to September 31, 1991			
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)			
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	K. Lin	Visiting Fellow	LMB, NCI
	K. Ashizawa	Visiting Fellow	LMB, NCI
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Mark Willingham, Chief, Ultrastructural Cytochemistry Section			LMB, NCI
LAB/BRANCH			
Laboratory of Molecular Biology			
SECTION			
Molecular Biology Section			
INSTITUTE AND LOCATION			
NCI, NIH, Bethesda, MD 20892			
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:	
4.0	3.0	1.0	
CHECK APPROPRIATE BOX(ES)			
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	
<input type="checkbox"/> (a1) Minors			
<input type="checkbox"/> (a2) Interviews		B	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)			
<p>To understand the molecular mechanism(s) by which thyroid hormone, 3,3',5-triiodo-L-thyronine (T₃) promotes growth, differentiation and development, the structure and activity of the cellular thyroid hormone binding proteins have been studied.</p> <p>I. Molecular interaction of T₃ with thyroid hormone nuclear receptor (TR). Using three monoclonal antibodies and immunocytochemistry, TR expressed and purified from <i>E. coli</i> was shown to be structurally identical to the endogenous TR. To determine the effect of domains A/B, C and D on the binding of T₃ to domain E, a series of deletion mutants of h-TRβ1 were expressed and purified from <i>E. coli</i>. Analysis of binding of these mutants to T₃ and its analogs indicated that domain E alone does not bind T₃. D domain is essential for T₃ binding and C domain has modulation activity. In contrast, A/B domain is not required for T₃ binding. Deletion analysis further showed that the last eight carboxyl amino acids are essential for T₃ binding. The T₃ binding site(s) in TR was covalently labeled with underivatized [3',5'-¹²⁵I]-L-thyroxine (T₄). The amino acids labeled with [¹²⁵I]T₄ were located in two tryptic peptides. The apparent molecular weight of the labeled peptides suggested that one of the two peptides could be the C-terminal peptide. These results are consistent with the deletion analysis in which the critical importance of the last eight C-terminal amino acids was demonstrated.</p> <p>II. Regulation of cytosolic thyroid hormone binding protein. The human p58-M₂ isolated from A431 cells is a monomer of pyruvate kinase, subtype M₂ (PKM₂). The effects of T₃ on enzymatic activity was evaluated using the active p58-M₂ expressed and purified from <i>E. coli</i>. T₃ inhibited the kinase activity of p58-M₂ in a dose-dependent manner. However, it has no effect on its association to the tetrameric PKM₂. Fructose-1,6-bisphosphate activated the conversion of p58-M₂ to PKM₂ <i>in vitro</i>. This mode of regulation also occurs <i>in vivo</i>. Dissociation of tetrameric PKM₂ to the monomeric p58-M₂ is a novel mechanism by which glycolysis shuts off in responding to glucose deprivation. The recombinant monomer of isoenzyme PKM₁ was also found to bind T₃ with affinity and specificity. Thus, cytosolic T₃ binding proteins belong to a multigene family probably functioning in a tissue-specific and developmentally-regulated manner.</p>			

Major Findings:I. Molecular interaction of T₃ with thyroid hormone nuclear receptor (TR).

A. By analogy with steroid hormone receptors, human placental thyroid hormone nuclear receptor (h-TRβ1) could be divided into four functional domains: A/B (Met¹-Leu¹⁰¹), C (Cys¹⁰²-Ala¹⁷⁰), D (Thr¹⁷¹-Lys²³⁷), and E (Arg²³⁸-Asp⁴⁵⁶). We have previously purified h-TRβ1 from *E. coli*. It retains hormone and DNA binding activity. However, it is uncertain whether the purified h-TRβ1 is structurally similar to the endogenous TR. We, therefore, compared the localization of the endogenous TR and transfected h-TRβ1 by immunocytochemistry. Three monoclonal antibodies (mAb J51, J52 and J53) were developed against the purified h-TRβ1. The epitopes for J51 and J52 were mapped to be in two different sites in the A/B domain. The epitope for J53 was localized in the E domain of h-TRβ1. Using J51 and J52, the endogenous TRβ1 in rat pituitary GH₃ cells was visualized to be exclusively present in nuclei. The transfected h-TRβ1 in monkey COS-1 and human choriocarcinoma JEG-3 cells were recognized by both J51 and J52. Interestingly, the intracellular localization of the transfected h-TRβ1 or rat TRβ1 in the above two cell lines depended on the level of expression. TRβ1 expressed at low levels was found exclusively in nuclei. However, for high level expression of TRβ1, cytoplasmic localization was also detected. J53, however, failed to detect nuclear fluorescence of the endogenous and transfected TRβ1 in fixed cells, indicating that its antigenic site is occluded. Localization of the endogenous and transfected TRβ1 in nuclei indicated that these two receptor proteins are structurally indistinguishable. Furthermore, the findings that TRβ1 could be localized in the cytoplasm when receptor was over-expressed suggested finite numbers of "acceptor" sites for TRβ1 in the nucleus.

B. Previously we have shown that T₃ binding site(s) was located in domain E. However, domain E alone did not bind T₃. To ascertain what other structural elements are required for domain E to function as a hormone binding domain, a series of deletion mutants were constructed. The mutants were expressed in *E. coli*, and the expressed proteins were purified. Analysis of the T₃-binding affinity and analog specificity of the purified truncated h-TRβ1 indicated that domain E alone did not have T₃-binding activity. Extension of the amino-terminal sequence of domain E yielded a mutant (Lys²⁰¹-Asp⁴⁵⁶) with a K_a for T₃ of 0.5 ± 0.2 × 10⁹ M⁻¹. Further extension to include the entire domain D (Met¹⁶⁹-Asp⁴⁵⁶) yielded a mutant which has T₃-binding activity with a K_a of 0.8 ± 0.1 × 10⁹ M⁻¹. Further extension of the amino-terminal sequence of domain E to include domain C increased the affinity for T₃ by nearly 2-fold (K_a of 1.5 ± 0.4 × 10⁹ M⁻¹). The K_a for the wild-type h-TRβ1 is 1.5 ± 0.2 × 10⁹ M⁻¹. Furthermore, mutant (Met¹⁶⁹-Asp⁴⁵⁶) binds to 3,3',5-triiodo-L-thyropropionic acid, D-T₃, L-thyroxine (T₄), and 3',5',3-triiodo-L-thyronine with 307%, 37%, 7% an 0.1%, respectively, of the activity of L-T₃. This order of analog affinity is similar to that of the wild-type h-TRβ1. These results indicate that domain D is essential for hormone-binding activity. In contrast, the A/B domain is not required for T₃-binding activity. Domain C has modulation activity for domains D and E. Deletion of the last eight carboxyl amino acids completely abolishes the T₃-binding activity of the mutant (Met¹⁶⁹-Asp⁴⁵⁶). Thus, domain D is essential for domain E to function as a hormone-binding domain.

C. To identify the amino acid residues which interact with T₃ in the hormone binding site, we affinity-labeled truncated protein of h-TRβ1 (KD29, Lys²⁰¹-Asp⁴⁵⁶) with underivatized [3',5'-¹²⁵I]-T₄. In the presence of 100-fold molar excess of unlabeled T₃, the labeling was inhibited completely, indicating that the labeling was specific. Digestion of the carboxy methylated and succinylated [¹²⁵I]-labeled KD29 yielded two [¹²⁵I]-labeled peptides. Comparison of the size of the labeled peptides with those of the expected peptides according to the deduced amino acid sequence suggested that one of the two labeled peptides could be the C-terminal peptide. These

results are consistent with the deletion analysis in which the critical importance of the last eight carboxyl terminal amino acids was demonstrated

II. Regulation of cytosolic thyroid hormone binding protein (CTHBP).

A. We have previously shown that the cytosolic thyroid hormone binding protein (p58-M₂) in human epidermoid carcinoma A431 cells is a monomer of pyruvate kinase, subtype M₂ (PKM₂). To further characterize the molecular properties of p58-M₂, we overexpressed p58-M₂ in *E. coli* and purified it to homogeneity. At 22° C, the monomeric p58-M₂ exhibited kinase activity with an apparent V_{max} of 22 ± 9 units/mg. The Km for adenosine diphosphate (ADP) and phosphoenol pyruvate (PEP) are 3.85 ± 2.4 and 1.55 ± 0.73 mM, respectively. These results indicated that p58-M₂ has intrinsic kinase activity. Analysis of the molecular size indicated that the activation of p58-M₂ by fructose-1,6-P₂ bisphosphate (Fru-1,6-P₂) resulted in the association of the monomeric p58-M₂ to the tetrameric PKM₂. p58-M₂ bound to T₃ (K_a = 1.7 × 10⁷M⁻¹) and exhibited analog specificity; whereas, PKM₂ did not bind thyroid hormone. The order of binding affinity was L-T₃>L-T₄>3,3', 5-triiodo-L-thyropropionic acid>3'-isopropyl-3,5-triiodo-L-thyronine>3',5',3-triiodo-L-thyronine. Binding of T₃ and its analogs resulted in the inhibition of the kinase activity of p58-M₂. The order of kinase inhibitory activity and preventing its association to tetrameric PKM₂ was parallel to that of binding activity. The present study demonstrated that *in vitro*, the molecular mechanism by which Fru-1,6-P₂ induced activation of PK activity is by facilitating the tetramer formation. Furthermore, thyroid hormone plays a role in regulating the enzymatic activity of PKM₂.

B. To evaluate whether the association of the monomeric p58-M₂ to the tetrameric PKM₂ is regulated by Fru-1,6-P₂ *in vivo*, we prepared monomer-specific monoclonal antibody and quantified the monomer formation *in situ* in cultured cells by immunocytochemistry. The intracellular Fru-1,6-P₂ was manipulated by the glucose concentration in the media. At the physiological concentration of glucose (4-6 mM), 30-35% of PK existed as a monomer. However, PKM₂ was dissociated into monomer within minutes after cells were deprived of glucose. The maximal level of monomer was detected after one hour at 37°C. Monomer was rapidly (within minutes) converted to tetramer after addition of glucose. Furthermore, when cells cultured in 10 mM of glucose were treated with cytochalasin B, an inhibitor of the glucose transporter, a maximal level of monomer was detected within 20-30 minutes. These results indicate that monomer-tetramer interconversion is a major *in vivo* cellular regulatory mechanism in response to changes in the extracellular glucose concentration.

C. CTHBP has been identified in tissues of many species and cultured cells. The binding constants of T₃ varied greatly from 6.6 × 10⁶ M⁻¹ for CTHBP from rat cerebellum to 3 × 10⁹M⁻¹ for CTHBP from human red blood cells. It is unclear, however, whether these variations reflect differences in the cytosolic preparations under different experimental conditions or differences in the identity of protein molecules from different species and tissues. Furthermore, in rat brain, liver and heart CTHBP was found to be developmentally regulated. In rat brain, the affinity of rat cerebellum CTHBP for T₃ is highest on day 10 when brain undergoes rapid differentiation and drops dramatically with age with little or no change in the binding capacity. In heart, similar changes in affinity were also found. It is unknown whether the changes reflected the differential regulation of different CTHBPs at different stages of development.

There are four mammalian isoenzymes of pyruvate kinase known, each of which consists of four identical or nearly identical subunits with the molecular weight of each subunit ranging from 57-60K. The four isoenzymes are designated as L, R, M₁ and M₂. The four isoenzymes are expressed in a tissue-specific and developmental manner.

Based on the above consideration, it is possible that the heterogeneity observed for the interaction of T₃ with CTHBP in different tissues and in different developmental stages could be due to the existence of different T₃ binding molecular species. We, therefore, evaluated whether the monomeric form of PKM₁ also binds T₃.

Using a T7 expression system, the monomer of rat pituitary pyruvate kinase, subtype M₁ (PKM₁), was overexpressed in *E. coli* and purified to homogeneity as indicated by SDS-polyacrylamide gel electrophoresis. Characterization of the purified protein by a sizing column indicated that it consisted of two molecular species: the major one is a tetrameric PKM₁ and the minor species is its monomeric form (p58-M₁). The maximal specific activity of the purified PKM₁ was 217 ± 13 units/mg. It exhibits hyperbolic kinetic properties with a K_m for ADP to be 0.70 ± 0.17 mM and for phosphoenol pyruvate (PEP), 0.025 ± 0.005 mM. The monomeric p58-M₁ has intrinsic enzymatic activity with a V_{max} of 79 ± 20 units/mg and K_m's for ADP and PEP of 1.43 ± 0.76 and 0.14 ± 0.07 mM, respectively. Furthermore, the monomer binds T₃ with K_a = 1.5 × 10⁷M⁻¹. The order of analog specificity is T₃ >L-T₄>3'-isopropyl-3,5-diiodo-L-thyronine >3',5',3-triiodo-L-thyronine. In contrast, tetrameric PKM₁ lacks T₃ binding activity. The enzymatic activity of p58-M₁ is inhibited by T₃ and its analogs in a concentration-dependent manner with the order of inhibitory activity similar to that of binding activity. This inhibition, however, is reversed by the addition of Fru-1,6-P₂. p58-M₁ is the second PK isoenzyme monomer to be identified as having thyroid hormone binding activity. These results indicate that cytosolic thyroid hormone binding proteins, like the T₃ nuclear receptor, belong to a multi-gene family, probably to function in a tissue-specific and developmentally-regulated manner.

Publications:

Kimura H, Noiva R, Mizunaga T, Yamauchi K, Horiuchi R, Cheng S-y, Lennarz WJ. Thyroid hormone binding protein contains glycosylation site binding protein activity, *Biochem Biophys Res Commun* 1990;170:1319-24.

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Ashizawa K, McPhie P, Lin K-H, Cheng S-y. An *in vitro* novel mechanism of regulating the activity of pyruvate kinase M_2 by thyroid hormone and fructose-1,6-bisphosphate, Biochem 1991, in press.

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

PROJECT NUMBER

Z01 CB 08753-09 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Immunotoxin and Oncotoxin Therapy of Cancer Cells

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

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Co-investigators:	D. FitzGerald	Microbiologist	LMB, NCI
	M.C. Willingham	Chief, UCS	LMB, NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

14.5

PROFESSIONAL:

12.0

OTHER:

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.)

Pseudomonas exotoxin (PE) or genetically modified forms of PE have been attached to monoclonal antibodies (mAbs) or growth factors to create cell-specific cytotoxic agents. Mutant PE molecules in which domain I has been replaced by growth factors, CD4 or single chain antibody combining regions have been created by gene fusion, the chimeric proteins produced in *E. coli*, and purified to near homogeneity. We have constructed and studied the activity of the following chimeric toxins: TGF α -PE40, IL2-PE40; IL4-PE40, IL6-PE40, IGF1-PE40, acidic FGF-PE40, CD4-PE40, anti-Tac(Fv)-PE40 and antitransferrin(Fv)-PE40. TGF α -PE40 which kills cells with EGF receptors has now been shown to have an antitumor effect in mice when injected I.P. against intraperitoneal tumors and against subcutaneous tumors. IL2-PE40 is very effective in killing mouse and rat cells with IL2 receptors but is less active against primate and human cells. To overcome this deficiency, a single chain immunotoxin anti-Tac(Fv)-PE40 was constructed which is extremely cytotoxic to human and primate cells containing IL2 receptors including cells directly isolated from patients with adult T cell leukemia. IL6-PE40 and a variant, IL6-PE66^{Glu}, is cytotoxic to several myeloma cells lines and hepatoma cell lines; cells with as few as 400 receptors per cell can be killed. Because tumors are dependent on a new blood supply, we constructed acidic FGF-PE40 and PE66^{Glu} which are cytotoxic to FGF receptor bearing cells. CD4-PE40 has been tested in combination with AZT and shown to act synergistically to arrest the spread of HIV infection in culture. A monoclonal antibody, B3, reactive with many colon, breast, lung and ovary tumors has been isolated, the genes encoding the variable regions cloned and a single chain immunotoxin made. B3(Fv)-PE40 has a strong antitumor effect against human tumors growing in nude mice and is currently undergoing preclinical development. *Pseudomonas* exotoxin mutants with increased activity have been created by changing the carboxy terminus from REDLK to KDEL. These molecules are two to ten-fold more cytotoxic to target cells. To permit prolonged therapy with immunotoxins which are very immunogenic, the effect of an immunosuppressive agent, 15-deoxyspergualin, has been studied and the drug shown to suppress primary antibody formation to PE in mice. Modified clones of PE have been constructed with foreign polypeptides inserted into the translocating domain of PE which introduces these inserts into the cytosol of target cells.

Other Personnel:

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L. Pai	Medical Staff Fellow	LMB, NCI
T. Prior	Visiting Fellow	LMB, NCI
R. Kreitman	Clinical Associate	LMB, NCI
S. Seetharam	Visiting Fellow	LMB, NCI
M. Gallo	Microbiologist	LMB, NCI
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U. Brinkman	Special Volunteer	LMB, NCI
C. Kasturi	Visiting Fellow	LMB, NCI
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Q.-C. Wang	Visiting Associate	LMB, NCI
V. K. Chaudhary	Special Volunteer	LMB, NCI

Major Findings:

Our previous structure-function analysis of *Pseudomonas* exotoxin had shown that domain I (amino acids 1-252) is the cell binding domain, domain II (amino acids 253-364) makes up the translocation domain, and domain III (amino acids 400-413) makes up the ADP-ribosylation domain. The function of amino acids within domain Ib (amino acids 365-399) is unknown and most of it can be deleted without affecting the activity of *PE*. We have employed the translocation and ADP ribosylating domains of *PE* (amino acids 253-613) (or slightly modified forms of it) to make immunotoxins either by attaching *PE40* by chemical linkage to targeting ligands such as antibodies, or to make chimeric toxins. The latter are made by fusing DNA elements encoding growth factors or antibody combining regions to the gene encoding *PE40*. The chimeric toxins are made and expressed in *E. coli* using a T7 polymerase based expression system and are purified from inclusion bodies to near homogeneity. Using this approach, we have made the following chimeric toxins: TGF α -*PE40*, IL2-*PE40*, IL4-*PE40*, IL6-*PE40*, IGF1-*PE40*, acidic FGF-*PE40*, and CD4-*PE40*. We also have synthesized several single chain immunotoxins in which the variable regions of antibodies in a single chain form are fused to *PE40*. These include anti-Tac(Fv)-*PE40*, anti-transferrin-Fv(*PE40*) and most recently B3(Fv)-*PE40*.

TGF α -*PE40* kills cells with EGF receptors. Because many cancers overexpress EGF receptors and often contain amplified EGF receptor genes, this molecule has been tested against tumors with high numbers of EGF receptors. We have examined several ways of administering TGF α -*PE40* and found continuous release by a pump implanted in the peritoneal cavity is the most effective route. Using this route, Pai et al. have shown that TGF α -*PE40* will cause regression of two subcutaneous tumors. One is an epidermoid carcinoma and the other is a prostate carcinoma. A modified form of TGF α -*PE40* in which the four cysteines in *PE40* have been mutated to alanines has been prepared by Merck & Co. under a license from NIH and is scheduled to be introduced into the clinic to treat bladder cancer in 1991.

IL2-*PE40* and anti-Tac(Fv)-*PE40* which kills cells bearing IL2 receptors was previously shown to be very effective in killing cell lines with IL2 receptors and in suppressing autoimmune diseases and allograft rejection in mice and rats. However, this molecule had very little activity against adult T cell leukemia cells directly isolated from patients or human T cells activated by lectins or MLR. To overcome this deficiency, several modified molecules were made, one of which is the single chain immunotoxin, anti-Tac(Fv)-*PE40* which reacts with the p55subunit of the IL2 receptor. This

molecule was shown to be very cytotoxic to cells directly isolated from patients with adult T cell leukemia as well as against monkey and human T cells activated in a MLR or by lectins. Several mutant forms of anti-Tac(Fv)-PE40 were constructed in order to produce a molecule which could be renatured more successfully to yield more active chimeric toxin. These included deletion of the second disulfide bond in domain II and conversion of cysteines to alanines in PE. None of these constructions resulted in a better yield of active toxin. In collaboration with Protein Design Labs., we are now trying to make enough anti-Tac(Fv)-PE40 for monkey allograft trials using transplanted hearts or kidneys.

IL6-PE40 was found to kill several myeloma cell lines and hepatoma cell lines as well as one prostate cancer cell line. In an effort to increase the activity of the IL6 receptor targeted agent, we have made several modifications in the PE molecule and made IL6 chimeric toxins using these mutant molecules. The most active molecule constructed is IL6-PE66^{4Glu} KDEL in which domain I has been retained and four inactivating mutations (Arg → Glu) inserted in domain I. In addition, the carboxyl terminus of PE was changed from REDLK to KDEL which has been shown to generally increase the activity of PE containing molecules two- to three-fold. IL6-PE66^{4Glu} KDEL is a very active molecule which can kill hepatoma cells with as few as 400 IL6 receptors per cell. The molecule has been tested in nude mice bearing a subcutaneous hepatoma and found to cause an anti-tumor effect in this model. In addition, IL6-PE66^{4glu} has been tested against cells obtained from the bone marrow of patients with multiple myeloma in collaboration with J. Epstein and B. Barlogie (University of Arkansas) and shown to be cytotoxic to about 70% of the samples from patients with multiple myeloma. Efforts are being made to find a company interested in developing this drug.

IGF1-PE40. Molecules cytotoxic to cells bearing IGF1 or IGF2 receptors were constructed by fusing a cDNA encoding IGF1 to PE40. These molecules have good cytotoxic activity even though the IGF1 molecule does not fold up properly so that its cell binding capacity is only 5% of that of native IGF-1.

Acidic FGF-PE40 and PE66. Because tumors are dependent on a new and expanding blood supply, we have constructed chimeric toxins directed at these FGF receptor containing cells. These chimeric toxins kill cultured endothelial cells and are also cytotoxic to several cancer cell lines with FGF receptors. Of the several chimeric toxins produced, α FGF-PE66^{4Glu}KDEL was found to be the most cytotoxic and is now being evaluated in animal models.

CD4-PE40 is a chimeric toxin in which the first 178 amino acids of CD4 were fused to PE40. This chimeric toxin binds to gp120 on HIV infected cells and subsequently kills these HIV-infected cells. Recently, the combined effect of CD4-PE40 and AZT was tested on cultures of recently infected T cells. The combination acted synergistically arresting the spread of infection and eliminating HIV from the culture. The IND application for CD4-PE40 has recently been submitted by the Upjohn Company and the drug should be evaluated in patients very soon.

Bispecific toxins. In an attempt to make chimeric toxins that are more specific or more active, we have constructed chimeric toxins with two (different or similar) binding domains. These include molecules such as TGF α -anti-Tac(Fv)-PE40 where TGF α and anti-Tac(Fv) are in tandem before PE40, and IL6-PE^{4Glu}-TGF α where TGF α is inserted just prior to the carboxy terminus of PE. The activity of these molecules on cultured cells and tumors is being studied.

Monoclonal Antibodies. Two new monoclonal antibodies, B3 and K1, that react selectively with certain cancers have been isolated this year. MAb B3 reacts with a carbohydrate present on the surface of many adenocarcinomas of the colon, lung, ovary, and breast and with only a few

were constructed by chemical means and found to kill antigen bearing cells in tissue culture and to cause complete regression of two different human carcinomas in mice. MAb B3 reacts with the same normal tissues (stomach, trachea and bladder) in humans and monkeys. Therefore, monkeys have been used to show that doses that are therapeutic in tumor bearing mice (25-50 µg/kilo) are tolerated by monkeys without bleeding from the stomach or bladder. On the basis of these studies, B3-PE and B3-PE40 are being developed for clinical use. MAb K1 reacts with an antigen (CAK1) that is expressed on many ovarian cancers, mesotheliomas, and esophageal cancers. However, the antigen is not well internalized and, therefore, makes a poor target for immunotoxins. The possibility of using MAb K1 for radioisotope therapy is currently being explored.

C242 is a MAb isolated by scientists at Pharmacia that reacts with many colon carcinomas and few normal tissues. C242 was conjugated to PE and PE40 and shown to make an active immunotoxin that produces regression of antigen positive tumors in mice. Efforts to make an active single chain immunotoxin are underway.

Pseudomonas Exotoxin Mutants with Altered Properties. An ideal immunotoxin or oncotoxin would have high specific cell killing activity, low nonspecific toxicity, a long half-life in the blood, and low immunogenicity. Several of these points are now being addressed. It was found that chimeric toxins with a two to ten-fold increase in activity were created by changing the carboxyl terminal sequence from REDLK to KDEL. To date, we have made TGF α -PE40KDEL, anti-Tac(Fv)-PE40KDEL, CD4-PE40KDEL and IL6-PE66^{4Glu}KDEL and all have the expected increase in activity. We believe that proteases such as thrombin and plasmin are responsible for the rapid degradation of chimeric toxins in the blood. Therefore, we have determined the cleavage sites for these proteases in PE and begun to make protease resistant mutants. One of these, PE Δ 490, has a 70% increase in its survival in the circulation of mice. The conjugation of polyethylene glycol (PEG) to proteins has been previously shown to reduce their immunogenicity. To direct PEG to sites which will not interfere with the cytotoxicity of a chimeric toxin, we have engineered a chimeric toxin (a derivative of TGF α -PE38) that only has lysine residues in a linker peptide between TGF α and PE38. The molecule is active and will soon be derivatized with PEG.

As an alternative approach to the problem of immunogenicity, the effect of an immunosuppressive agent, 15-deoxyspergualin (DSG) was evaluated in mice treated with immunotoxins. DSG was found to completely suppress the production of antibodies to PE in mice. The effect of this drug in monkeys is currently under investigation.

Previous studies have shown that amino acids 280-613 of PE are translocated across a cellular membrane into the cytosol. We have explored the translocation function of domain II by inserting foreign polypeptide sequences into different nonessential regions of this fragment. We have inserted a ribonuclease (barnase), near the carboxyl end of PE and shown it is translocated into the cytosol where it degrades cellular RNA. We have also inserted somatostatin and other peptides into domain Ib and shown these are also translocated. We are examining this approach to determine if it may be used to introduce antigens into the cytosol of T cells for presentation by MHC Class I.

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

PROJECT NUMBER

Z01 CB 08754-08 LMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
13.0	13.0	0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The simultaneous resistance of cancer cells to many different anti-cancer drugs is the major impediment to successful chemotherapy of metastatic disease. An important mechanism of multidrug resistance is expression of P-glycoprotein, a 170,000 dalton energy-dependent drug efflux pump which removes natural product drugs from the cell. We have continued our studies of multidrug resistance by an analysis of the mechanism by which this pump removes drug from within the plasma membrane or from the cytoplasm. In an *in vitro* vesicle system, ATP has been shown to be the preferred energy source, and many of the drugs which are transported compete with each other for a single site or small number of sites on the transporter. Labeling sites for the P-glycoprotein inhibitor ³H-azidopine occur in both the amino and carboxy-terminus of the protein, and these two sites appear likely to make up the single channel through which the drugs move. Molecular manipulations have identified the first intracytoplasmic loop as a domain involved in drug recognition, which is distinct from the drug labeling sites identified with ³H-azidopine. We have also developed an *MDR1* transgenic mouse whose bone marrow is protected from the cytotoxic effects of anti-cancer drugs by expression of P-glycoprotein. This model can be used to identify potent agents which inhibit the multidrug transporter *in vivo*, since these agents sensitize the transgenic mice to the leukopenia induced by chemotherapy. The *MDR1* cDNA can also be introduced into mouse bone marrow by retroviral infection. Such *MDR1* retroviral vectors should be useful for gene therapy to protect bone marrow during cancer therapy and to introduce non-selectable genes into bone marrow. New *in vitro* models of resistance to VP-16 and *cis*-platinum, not involving the multidrug transporter, are under development.

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Major Findings:

(1) Cells selected for resistance to natural product chemotherapeutic drugs in culture often contain amplified copies of the *MDR1* gene. In human KB carcinoma cells, these amplified copies exist on episomes ranging in size from 700 kb to several thousand kb, which can be directly demonstrated after gamma irradiation on pulsed field gradient gels. The evolution of the episomes in one multidrug resistant cell line which had been selected in colchicine has been studied in detail. The amplified *MDR1* gene first appears on an episome of approximately 700 kb, at which time only "minute" chromosomes are visible cytogenetically. The next step in amplification involves a doubling in size of this episome, coincident with the appearance of "double minute" chromosomes. Selection to higher levels of drug resistance in this cell line results in alterations in size of the "double minute" chromosomes, and is consistent with the presence on the original episome of a gene other than the *MDR1* gene whose amplification is deleterious to the cell. Other multidrug resistant cell lines have different patterns of evolution of their extrachromosomal DNA, including multidrug resistant cells in which the primary event appears to be formation of an episome of several thousand kb.

(2) We have continued to characterize a model system in which energy dependent drug transport can be demonstrated in inside out vesicles derived from multidrug resistant cells. In this system, using ^3H -vinblastine as a primary substrate for transport into the vesicles, several other drugs handled by the transporter appear to compete for the transport site. These results suggest that there are only a small number, and possibly only one, transport site for the many different drugs which are pumped by P-glycoprotein. By testing a large number of nucleotide phosphates as potential sources of energy for the pump, we have shown that ATP is the preferred energy source. A three compartment kinetic model, which allows calculation of kinetic parameters associated with transport of drugs by the multidrug transporter across epithelial cell monolayers, has also been developed.

(3) The drug binding sites in P-glycoprotein have been localized using ^3H -azidopine as a ligand, various means of digesting P-glycoprotein into fragments and specific antibodies to identify the labeled fragments. P-glycoprotein has been digested using trypsin, Staph V8 protease, and cyanogen bromide. Antibodies have been generated against most of the intracellular domains of P-glycoprotein using P-glycoprotein fragments synthesized in *E. coli* as antigens. To do this, cDNAs encoding P-glycoprotein fragments were linked to

Pseudomonas toxin to produce highly immunogenic chimeric peptides. The immunoprecipitated ³H-azidopine-labeled P-glycoprotein fragments correspond to regions including the transmembrane domains of both the amino-terminus and the carboxy-terminus of P-glycoprotein. ³H-azidopine labeled can be inhibited by addition of vinblastine, which competes for the labeling sites. Both the amino-terminal and the carboxy-terminal labeling are equally inhibited by vinblastine, implying that both sites have equivalent affinity for both azidopine and vinblastine. These results suggest that the two labeled sites probably represent two halves of a single drug channel.

(4) By genetic manipulations, we have shown that the first cytoplasmic loop in P-glycoprotein contains determinants essential for drug recognition. During the selection of multidrug resistant cells in colchicine, high level resistance to colchicine resulted from substitution of a valine for glycine at position 185. Position 185 is in the first cytoplasmic loop of P-glycoprotein. We have made chimeras replacing this loop encoded by the *MDR1* gene with the homologous loop from the *MDR2* gene. These chimeras are non-functional with only 17 amino acids different out of a total of 89 in this region. Replacement of some of the *MDR2* amino acids in this region with the homologous amino acids from *MDR1* has resulted in a functional transporter, with some differences in substrate specificity. These results suggest that the first intracytoplasmic loop contains determinants for substrate specificity, despite the fact that the sites labeled by ³H-azidopine are not included within this loop.

(5) We have continued to develop the *MDR1* transgenic mouse as a model for testing agents which reverse drug resistance. In this model, mouse bone marrow, which expresses the human *MDR1* gene, becomes resistant to leukopenia induced by natural product anti-cancer drugs. Sensitivity to these drugs can be restored by agents, such as verapamil, quinidine, quinine, and cyclosporine A, which inhibit activity of the multidrug transporter. This transgenic mouse model enables the rapid screening of agents which can then be used in clinical trials to inhibit the multidrug transporter, thereby sensitizing drug resistant cancers to chemotherapy.

(6) It is possible to transfer multidrug resistance to cultured cells with a retroviral vector carrying the *MDR1* gene. We have shown that mouse bone marrow cells infected with this virus also become resistant to drugs such as colchicine and vinblastine, as detected in a GM-CFU assay *in vitro*. These infected cells can be introduced into mice where they form splenic foci which contain the *MDR1* gene. Since we have demonstrated that bone marrow expressing a transgenic *MDR1* gene can give a selective advantage to mice into which it has been transplanted, *MDR1*-retrovirus infected marrow should be selectable in mice. These studies form the basis for gene therapy of human cancer in which bone marrow can be protected from the cytotoxic effect of anti-cancer drugs, and in which the *MDR1* cDNA is used as a co-selected marker to introduce other genes into bone marrow.

(7) Many human cancers express the *MDR1* gene at levels comparable to those which give several-fold resistance *in vitro* and in our transgenic mice. Some human adult acute non-lymphocytic leukemias express such levels of P-glycoprotein. When expression of P-glycoprotein is found, poor response to chemotherapy including daunorubicin and cytosine arabinoside is predicted. These results suggest either that expression of P-glycoprotein is itself responsible for poor response, presumably because the pump protects the cells from daunorubicin, or that expression of P-glycoprotein is a reporter for other changes in the cell that result in increased malignancy. Clinical trials using agents that reverse multidrug resistance will be needed to distinguish these two possibilities.

(8) Some tumor populations express very low levels of P-glycoprotein, and it has been uncertain whether this result reflects a small percentage of more positive cells, or a generally low level of expression in all cells. To distinguish these possibilities, we have developed a technique for the isolation of cells which express P-glycoprotein on their surfaces from mixed populations containing positive and negative cells. This technique employs ferromagnetic beads coated with antibodies to P-glycoprotein which allow the magnetic sorting of as little as 1% positive cells from a negative population. Preliminary results in which lymphoma cells from previously treated patients were analyzed, suggests that lymphoma samples from these treated patients have a mixed population of highly positive and negative cells. This magnetic sorting technique can also be used to isolate positive cells from a mixed population in order to study the mechanism of increased expression of P-glycoprotein.

(9) We have begun to isolate cell lines which are drug-resistant for reasons other than expression of P-glycoprotein. Three different cell lines are currently being characterized:

- (1) Human melanoma (FEM-X) cells selected for resistance to VP-16 in the presence of a tiapamil analog which inhibits the multidrug transporter;
- (2) Human hepatoma cells selected in *cis*-platinum. Prior to selection, human hepatoma lines show a multidrug resistant phenotype which is unrelated to expression of P-glycoprotein. Selection in *cis*-platinum should allow identification of novel mechanisms for intrinsic resistance in this important human cancer;
- (3) Human KB carcinoma cells selected for high levels of resistance in *cis*-platinum.

(10) *mdr* RNA levels in rodent cells, but not in human cells, can be increased by treatment with cytotoxic drugs. This increase in *mdr* RNA results in increased P-glycoprotein and increased drug resistance. There is an associated increase in transcription as measured by nuclear run-off; suggesting that increased *mdr* mRNA levels result from mRNA stabilization.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08756-04 LMB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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
	M. C. Willingham	Chief, Ultrastructural Cytochemistry Sect. LMB, NCI
	C. Jhappan	Visiting Associate LMB, NCI
	G. Mickisch	Special Volunteer LMB, NCI
	H. Takagi	Visiting Fellow LMB, NCI
	R. Sharp	Biologist LMB, NCI
COOPERATING UNITS (if any)		
G. Smith and R. Callahan, Laboratory of Tumor Immunology and Biology, DCBDC, NCI; N. Fausto, Laboratory of Pathology & Laboratory Medicine, Brown University		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Biology Section		
INSTITUTE AND LOCATION NIH, NCI, Bethesda, MD 20892		
TOTAL MAN-YEARS 5.0	PROFESSIONAL: 4.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)		
<p>The advent of transgenic technology, in which foreign genetic information is stably introduced into the mammalian germ line, has provided a powerful approach to the study of gene function and regulation. We are continuing to use this technology to investigate several important aspects of cancer pathogenesis: the role of growth factors, receptors and oncogenes in the initiation and development of neoplasia, and the ability of malignantly transformed cells to evade destruction by chemotherapeutic agents.</p> <p>Transforming growth factor alpha (TGFα) and epidermal growth factor (EGF) stimulate cellular proliferation by binding and activating the EGF receptor tyrosine kinase. Perturbation of this signal transduction pathway can transform cells in culture, and has been implicated in the development of human cancer. To examine this hypothesis <i>in vivo</i>, transgenic mice were made bearing either the human TGFα or EGF receptor gene. TGFα overexpression induces hepatocellular carcinoma, mammary adenocarcinoma, pancreatic metaplasia and fibrosis, and gastric cystic hyperplasia. Furthermore, analysis of these transgenic mice has led to new discoveries about normal development of the breast and testis. Transgenic mice made using an activated form of a related gene, int-3, which contains EGF repeats and is a member of the Notch gene family, developed hyperplasia of secretory epithelia and neoplasia of the salivary and mammary glands. In addition, male mice were sterile and female mice could not lactate.</p> <p>The human <i>MDR1</i> gene encodes a multidrug transporter capable of conferring multidrug resistance to malignant cells. Bone marrow suppression is a major impediment to the use of chemotherapy in cancer treatment. Transgenic mice have been made that express the <i>MDR1</i> gene in the normally drug-sensitive bone marrow. The transgenic marrow becomes resistant to the cytotoxic effects of a number of commonly used chemotherapeutic drugs. These transgenic mice have been used as a model to test the efficacy of new chemotherapeutic agents, including reversing agents that can sensitize malignant cells and cure previously unresponsive cancers.</p>		

Major Findings:

Transgenic mice are made in this laboratory by the microinjection of purified DNA fragments into pronuclei of single-cell mouse embryos derived from inbred FVB/N mice. Embryos surviving this procedure are transferred into pseudopregnant CD1 foster mothers. Founder transgenic pups are identified by Southern blot analysis of tail genomic DNA. Mice possessing transgenes in their germ line are expanded to establish heterozygous lines, and heterozygotes are often mated to generate homozygous animals. Transgenic mouse lines established using this approach have been instrumental in our quest to elucidate the function and regulation of specific genes.

We and others have shown that overexpression of the EGF receptor and its ligands can transform cultured cells. To determine the *in vivo* consequences of perturbing the EGF receptor signal transduction pathway, we made transgenic mice using TGF α and EGF receptor genes. In one series of experiments, a DNA fragment containing the human TGF α cDNA driven by the mouse metallothionein-1 promoter was microinjected into one-cell embryos. Mice bearing this transgene expressed human TGF α RNA and protein in the majority of tissues tested, including the liver, pancreas, stomach and breast. Elevated levels of TGF α were detected in the blood and urine of transgenic mice, reminiscent of studies on cancer patients. TGF α transgenic mice were characterized by the progressive development of a number of dramatic lesions. A high incidence (75%) of liver tumors was demonstrated in male transgenic mice over ten months old, and mammary adenocarcinoma was observed in multiparous female mice. In addition, all mice developed severe cystic adenomatous hyperplasia of the stomach, and florid ductular metaplasia with associated interstitial fibrosis of the pancreas. Furthermore, abnormal development of the mammary gland was noted in TGF α transgenic mice. Using a transgenic approach we have demonstrated that TGF α contributes to the development of these lesions; however, it may not be sufficient. Experiments are underway to identify other causative agents, and to define the molecular mechanisms of pathogenesis. We believe that the TGF α transgenic mice will prove to be excellent models for a number of serious human diseases.

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 in the testis, but not in other tissues, suggesting that the transgene integrated near a gene containing a testis-specific enhancer element. *In situ* RNA data indicated that the EGF receptor transgene was being transcribed early in meiosis, while immunohistochemical analysis demonstrated that human EGF receptors first appeared postmeiotically at the late spermatid stage of spermiogenesis. This showed that the EGF receptor transgene was being regulated by a translational control mechanism. At spermiation EGF receptors were sequestered in residual bodies, and excluded from the sperm by some efficient compartmentalization mechanism. Ultimately, receptors in the residual bodies were degraded by the Sertoli cells. When homozygous mice were generated from this line, only male mice were found to be sterile. The sperm isolated from sterile homozygotes were paralyzed. Electron microscopic analysis of the tails of these sperm demonstrated that the axonemes were composed of an abnormal configuration of peripheral microtubules (5+2 instead of the normal 9+2). Furthermore, this unusual axonemal structure was found in the epididymis and vas deferens, but not in the testis, suggesting that microtubules were assembled normally, but possessed a latent instability. Axonemal aberrations of this type have been observed in the sperm of sterile men. This unique transgenic mouse line should prove to be valuable in the study of male sterility, microtubule stability, spermatogenesis-specific gene expression, translational control, sperm membrane specialization and testicular receptor degradation.

We also are using transgenic mice to study growth factors and receptors more distantly 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 to integrate into and activate the int-3 gene, which contains repeats similar to the yeast CDC10 cell cycle start protein and EGF repeats. We used a DNA fragment containing the activated int-3 gene isolated from a mouse breast tumor to generate transgenic mice. The phenotypes which developed in these transgenic mice were striking. Severe hyperplasia was associated with numerous secretory epithelial tissues, including mucosal glands, lacrimal glands, salivary glands and the epididymis. In addition, female and male mice develop undifferentiated mammary and salivary adenocarcinomas at a very high frequency. All int-3 male mice were sterile, and int-3 females were characterized by abnormal mammary gland development, which prevented lactation. Despite this, a single int-3 line was established. The precise relationship between int-3 and these dramatic phenotypic manifestations are being examined in this transgenic line. We hope to enhance our knowledge of the molecular pathogenesis of mammary gland neoplasia by mating TGF α and int-3 mice and analyzing the resulting doubly transgenic mice.

To determine the transcriptional mechanisms by which protooncogenes are regulated, the EGF receptor gene promoter has been isolated and characterized using protein-binding assays, mutagenesis analysis, and a cell-free transcription system (see Annual Report Number Z01 CB 08000-21 for details). To validate extensive information obtained by transfection assays, and to examine *in vivo* promoter function, DNA fragments containing all *cis* elements required for optimal activity in transfected cultured cells were used to drive the expression of the chloramphenicol acetyltransferase (CAT) reporter gene in transgenic mice. Unexpectedly, in all eight expressing lines of mice the EGF receptor gene promoter was most active in the thymus, based on quantification of CAT activity in transgenic mouse tissues. Cell separation techniques were used to demonstrate that the EGF receptor promoter was not active in the thymocytes, but only in the stromal tissue containing thymic epithelial cells. These intriguing results raise the possibility that the EGF receptor may play an important role in thymic epithelial cell function and/or thymocyte differentiation. In addition, this novel promoter can be used to target expression of various transgenes to the thymic epithelium.

The ability of malignant cells to evade destruction by chemotherapeutic agents has proven to be a major obstacle to effective cancer therapy. A human gene (*MDR1*) has been isolated in this laboratory encoding a 170 kDa glycoprotein (called P-glycoprotein) that is capable of conferring multidrug resistance to animal cells (see Annual Report Number Z01 CB 08754-08 for details). Transgenic mice were generated carrying a DNA fragment in which the human *MDR1* cDNA was driven by the chicken β -actin gene promoter. Human *MDR1* RNA and P-glycoprotein were detected in the transgenic bone marrow, which in nontransgenic animals is exquisitely sensitive to chemotherapy. To determine if the bone marrow becomes drug resistant, these transgenic mice have been exposed to a broad range of chemotherapeutic agents in the *mdr* family including daunomycin, doxorubicin, taxol, actinomycin D, vincristine and vinblastine. The data clearly demonstrated that the transgenic bone marrow became 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 was defeated by the administration of appropriate reversing agents, such as verapamil. These *MDR1* transgenic mice are being used 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.

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

PROJECT NUMBER

Z01 CB 08757-04 LMB

PERIOD COVERED

October 1, 1990 to September 31, 1991

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

Development of Immunotoxins for Cancer

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

PI:	D.J. FitzGerald	Microbiologist	LMB, NCI
Other:	I. Pastan	Chief, Laboratory of Molecular Biology	NCI
	C. Fryling	Biologist	LMB, NCI
	M. Chiron	Visiting Fellow	LMB, NCI
	C. Siegall	Staff Fellow	LMB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

2.2

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Pseudomonas exotoxin (PE) is cleaved by cells to produce fragments of 28 kD, from the N-terminus, and 37 kD, from the C-terminus. This step is necessary to generate the C-terminal fragment which contains ADP-ribosylating activity and is translocated to the cytosol. Mutant forms of PE that cannot be cleaved, are not translocated to the cytosol and are not toxic. The protease responsible for cleavage is a membrane-associated enzyme and its activity can be detected in crude membranes from L929 cells. The pH optimum for cleavage is 5.5. Cells were fractionated on Percoll gradients and the proteolytic activity was found in fractions corresponding to endosomes but not in those containing lysosomes. The cleavage of PE by membranes is stimulated by divalent cations. The addition of EDTA completely inhibits the generation of the fragments. However, inhibitors of serine proteases and other inhibitors of metalloproteases do not. Membrane preparations cannot cleave PE276G, a mutant form of PE previously shown to be nontoxic because cells failed to generate a 37 kD fragment. Protease activity can be solubilized using non-ionic detergents (NP-40, Octyl-glucoside). Fractions with proteolytic activity have been recovered after ion exchange and gel filtration chromatography. The site of cleavage within the PE molecule is an arginine-rich loop, close to arginine 279. Mutagenesis of the PE gene close to arginine 279 has located residues that are needed for proteolysis and toxicity. Mutagenesis at the C-terminus of domain II has located residues important for translocation. A PE-binding protein, thought to be the PE-receptor, has been identified. While the receptor is known to be present in crude membranes and detergent extracts, its role in the proteolysis step is currently not understood. To understand further the biologic effect of the arginine to glycine mutation at position 276, TGF α -PE40276G was produced and tested for cytotoxic activity.

Major Findings:

Characterization of a Cellular Protease That Cleaves PE

Background:

PE binds and enters cells by receptor mediated endocytosis and is then cleaved by a cellular protease. Cleavage, followed by reduction, is necessary to generate an active toxin fragment which translocates to the cytosol and inhibits protein synthesis. Cells cleave PE to produce two prominent fragments, one of 28 kD and the other of 37 kD. The 28 kD fragment is derived from the N-terminus and is composed of all of domain I and a small portion of domain II. The 37 kD fragment, which is from the C-terminus, is composed of most of domain II and all of domain III and is the fragment that translocates to the cytosol. Mutant forms of PE that are not cleaved appropriately, such as PE with a change of arginine to glycine at residue 276 or PE with a change of arginine to glycine at residue 279, are not translocated and are non toxic. Based on these results and other data, cleavage is thought to occur in an arginine rich loop near the beginning of domain II. Besides the fragments of 28 and 37 kD, other fragments of 25 and 18 kD are produced. These are thought to be breakdown products of the 28 kD fragment.

Characterization of Cellular Protease

To understand the proteolysis step, a characterization of the cellular protease has been initiated. By analyzing cell fractions it was found that the protease was a membrane-associated enzyme. Its activity could be detected in crude membranes from L929 cells. The pH optimum for cleavage was found to be at or around 5.5. Cells were fractionated on Percoll gradients and the proteolytic activity was found in fractions corresponding to endosomes. None of the activity was found in fractions containing lysosomes. The cleavage of PE by membranes was stimulated by divalent cations. The addition of EDTA completely inhibited the generation of the fragments. However, inhibitors of serine proteases and other inhibitors of metalloproteases did not. Membrane preparations cannot cleave PE276G, which, as mentioned above, is non toxic because it cannot be cleaved by cells to generate the appropriate 37 kD fragment. Proteolytic activity can be solubilized from crude membranes using non-ionic detergents (NP-40, Octyl-glucoside). Octyl-glucoside solubilized material has been subjected to anion exchange, cation exchange, hydroxylapatite and gel-filtration chromatography. While fractions with proteolytic activity have been recovered after ion exchange and gel filtration chromatography, it has not yet been possible to obtain pure protease.

Protease purification

Protease purification will depend on the stability of the enzyme, our ability to separate it from other cellular components, the development of a quantitative assay for toxin cleavage (to measure accurately the specific activity of the enzyme), a source of abundant enzyme (preparing large number of tissue culture cells is too cumbersome) and the discovery of any co factors that may influence proteolysis. Preliminary data indicate that the enzyme, in crude membranes or extracts, is stable at 4°C for at least 10 days. Membranes can be stored in the freezer without loss of activity. Densitometric scanning of autoradiograms will be used initially to quantitate the proteolysis of radiolabeled PE. If this method proves to less than adequate, direct counting of excised bands will be performed. Recently, we have detected the appropriate proteolytic activity in rat liver membranes. Rodent liver will be used as the source of protease instead of tissue culture cells. Finally, along with the protease, the PE receptor has been detected in detergent solubilized material from crude membranes. PE binds its receptor with greater affinity at pH 5.5 than at pH 7.0. The receptor does not appear to have proteolytic activity, but may influence proteolysis by making bound toxin a better or worse substrate for the protease. The influence of the receptor on the degree of proteolysis will be examined.

Site of Cleavage

PE is proteolytically processed by cells to produce defined fragments of 28 and 37 kDa. Several approaches are possible to determine the exact site of cleavage. One is to obtain the 37 kDa fragment directly from toxin-treated cells and perform an Edman degradation on the recovered material. To date, even using immunoprecipitation techniques, it has not been possible to obtain enough material for conventional microsequencing. Two alternative approaches have been devised.

PE can be metabolically labeled by adding radioactive amino acids to the growth medium. This has been done with ^3H -leucine and ^{35}S -methionine. Because all the naturally occurring methionine residues are located near the N-terminus of PE, only full sized PE and the 28 kDa fragment, which is derived from the N-terminus, can be immunoprecipitated as labeled proteins. However, if by site-directed mutagenesis, methionine residues are introduced into the C-terminal portion of the toxin, then both 28 and 37 kDa fragments can be recovered as labeled fragments. By placing methionine residues on either side of the residue that is cleaved it has been possible to locate the residues involved in cleavage. The probable involvement of arginine 279 in the cleavage step has been determined using this technique. When a methionine residue was substituted for glycine at position 280 and radiolabeled toxin produced, the 37 kD fragment could be recovered from L292 cells as a labeled fragment. If the methionine was substituted for wild type amino acids at positions 277-279 the following results were noted. When methionine was placed at residue 277, proteolysis was normal but only full-sized PE and the 28kD fragment could be immunoprecipitated as labeled fragments. When methionine was substituted for proline at position 278, the amount of proteolysis was reduced considerably and only PE and the 28 kD fragment were labeled. When methionine was substituted for arginine 279, there was no proteolysis. Thus it appears that cleavage occurs either immediately before or after arginine 279. Experimentation with methionine labeling has not been able to distinguish between these two possibilities. Also, it must be considered that the substitution of methionine for wild type residues could alter the cleavage process. Direct sequencing of the N terminus of the 37 kD fragment would be best.

A more sensitive method of direct sequencing has been devised. It is possible to radiolabel PE metabolically to a high specific activity ($4\ \mu\text{Ci}/\mu\text{g}$) by adding ^3H -leucine to minimal growth medium. Also, a leucine auxotroph (strain BL21) has recently been generated to increase the likelihood of obtaining PE at an even higher specific activity. Radiolabeled PE will be added to cells for various incubation periods at 37°C . The 37 kD fragment will be recovered by immunoprecipitation. Since direct sequencing has not been sensitive enough to determine the N terminus, each cycle from the sequenator will be counted in the scintillation counter. The first leucine after arginine 279 is at residue 284. This should be close enough to detect a strong radioactive signal from column fractions and, by knowing the cycle number, the site of cleavage can be determined.

Characterization of PE receptor*

Detergent extracts of crude L929 membranes were subjected to SDS-PAGE. Gels were then transblotted to nitrocellulose and the blots probed with PE or PE mutants. PE was shown to bind to a high molecular weight component ($>200\ \text{kD}$) while PE mutants that are deficient in binding (to cells) did not bind. Binding was detected using a peroxidase labeled second antibody. While this technique is not the best for quantitation it was of interest to note that the strength of peroxidase stain was increased when the pH was lowered from 7.0 to 5.5. Thus, the PE receptor and the cellular protease that cleaves PE are both contained in crude membranes and detergent extracts and both appear to function at pH 5.5.

*The term receptor is used to denote a PE binding component that is likely to serve as the surface receptor. Additional experimentation is needed to confirm this.

Properties of TGF α -PE40276G

Because PE with a glycine at position 276 is nonoxic and cannot be cleaved by cells, it was of interest to examine the effect of introducing this mutation into a chimeric toxin. TGF α PE40276G was constructed and compared with TGF α PE40 for toxicity on cells possessing EGF receptors. On some cell lines (e.g. KB) TGF α PE40276G was 10-100-fold less active than TGF α PE40 but, surprisingly, on others (HT-29, OVCAR) their activities were quite similar. The toxicity for mice of TGF α PE40276G was 25-fold less than TGF α PE40. Thus, TGF α PE40276G may a valuable reagent for treating certain tumors that can process PE40 with this mutation at position 276. Cells cannot cleave PE276G, but treatment of this mutant with trypsin introduces a clip which converts it to an active toxin. Certain tumors produce trypsin-like enzymes that could activate TGF α PE40276G and so add another layer of selectivity to this kind of reagent.

Sequences in Domain II that may contribute to Translocation

Domain II is thought to play an essential role in translocating PE to the cytosol. The mechanism by which this occurs is unknown. However, the identification of key residues in this domain should help our understanding of the process. Important residues have been identified at two different locations. Cleavage by cells generates the 37 kD fragment that is likely to have GWEQL.... as its N-terminal sequence. When substitution mutagenesis was performed, it was found that residues 280-284 could be changed from wild type to methionine without a change in their susceptibility to cleavage by the cellular protease and, with one exception, these substitutions caused no diminution of toxic activity. However, when methionine (or other) is substituted for tryptophan 281, toxicity is abolished. Since this residue is at the N-terminus of the toxic fragment it is possible that it is directly involved in translocation.

Mutations were also made at the C-terminus of domain II. The functional boundary of domain II was established by showing that the last 19 amino acids (346-364) could be deleted from this domain without loss of toxicity. Thus amino acids 253-345 are essential for activity. In addition, certain amino acids appeared to be key for function. Amino acids 339 and 343 could not be altered while residues 344 and 345 could be changed without loss of toxic activity. When alanine 343 is changed to glutamine, cytotoxicity is lost, despite the fact that the toxin is proteolytically processed correctly. This residue and 339 is thought to be important for translocation since the defect is after the processing step and before ADP ribosylation.

Publications:

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LABORATORY OF CELL BIOLOGY
SUMMARY REPORT
DCBDC, NCI
OCTOBER 1, 1990 TO SEPTEMBER 30, 1991

The Laboratory of Cell Biology consists of the Molecular Cell Genetics Section (Michael M. Gottesman, Chief) and the Chemistry Section (Ettore Appella, Chief). The Chief of the Laboratory of Cell Biology, Michael Gottesman, oversees projects encompassing five major areas: (1) Resistance of cancer cells to anti-cancer drugs; (2) Effects of cAMP and p53 on regulation of cancer cell growth; (3) The role of surface antigens and proteases in the invasiveness and metastasis of cancer cells; (4) The mechanism of energy-independent proteolysis; and (5) The mechanism of antigen processing. Approximately 28 personnel working on 7 specific research projects have contributed to the progress outlined in this summary.

Resistance of Cancer Cells to Anti-Cancer Drugs

The major mechanism of intrinsic and acquired resistance to multiple natural product chemotherapeutic drugs is the expression of the *MDR1* gene, which encodes a 170,000 dalton membrane glycoprotein which acts as an energy-dependent drug efflux pump (P170, P-glycoprotein, or multidrug transporter). Michael Gottesman, Ira Pastan (Chief, Laboratory of Molecular Biology) and their co-workers have studied the mechanism of action of this efflux pump by determining which sites on the transporter are labeled by ³H-azidopine, and by a mutational analysis of the transporter. These studies indicate that there are two labeling sites on the transporter, believed to represent the two halves of the transporter which come together to form a "channel" through which drugs move, and that the first intracytoplasmic loop of the transporter contains information involved in drug recognition. Basic recognition of multiple, chemically unrelated substrates appears to occur within the plasma membrane. ATP is the preferred energy source for transport, as indicated by studies using vesicles prepared from multidrug resistant cells.

An *in vivo* model system, in which the human *MDR1* cDNA is expressed in the bone marrow of transgenic mice, has shown that levels of expression of *MDR1* RNA comparable to those found in many human cancers are sufficient to confer drug resistance on bone marrow. This model has allowed the testing of many potential inhibitors of the multidrug transporter, because active agents sensitize the bone marrow of the MDR-transgenic mice to chemotherapeutic drugs. Functional drug resistant bone marrow may be transferred from resistant to sensitive animals, demonstrating the potential utility of the *MDR1* cDNA as a dominant selectable marker in human gene therapy experiments. Retroviral vectors which allow expression of the human multidrug transporter in transformed bone marrow and muscle provide models for protecting normal human tissues against the toxic effects of chemotherapy, and for the use of the *MDR1* cDNA in co-transformation of non-selectable genes.

Effects of cAMP and p53 on Cancer Cell Growth

cAMP stimulates the growth of certain epithelial cells and inhibits the growth of lymphoid and fibroblastic cells. Michael Gottesman's group has demonstrated that mutations in cAMP-dependent protein kinase, or increased expression of cyclic nucleotide phosphodiesterase, ablate growth responses to cAMP. The ability to create moveable genetic elements (either mutant protein kinases or phosphodiesterase expression vectors) makes it possible to study the

role of cAMP in growth regulation and gene expression of a variety of differentiated cell types. Two novel cAMP-dependent protein kinase mutants have been generated *in vitro* by site-directed mutagenesis, and their effect on activity of cAMP-dependent protein kinase synthesized in *Escherichia coli* has been analyzed.

p53 protein is another major factor which regulates growth of normal and cancer cells. Stephen Ullrich and Ettore Appella have found that wild-type p53 arrests cells in late G1 concomitant with a reduction in p34^{cdc2} kinase and PCNA, two proteins thought to be involved in cell cycle progression out of G1. p53 mutations have been found in several human malignancies, including Raji B lymphoma, and lung cancers. However, elevated wild-type p53 was found in Gardner's syndrome, and some lung cancer cells, without an anti-proliferative effect. These results suggest that other cellular factors may control levels of p53 in both normal and tumor cells.

The Role of Surface Antigens and Proteases in the Invasiveness and Metastasis of Cancer Cells

Vincent Hearing has studied the normal process of melanogenesis and surface antigens on melanoma cells which affect immunogenicity. Three different tyrosinases associated with pigment formation in the mouse have been isolated and characterized. The product of the *albino* locus appears to be the major determinant of melanogenesis in the mouse, whereas the product of the *slaty* locus encodes a tyrosinase with a distinct catalytic specificity, and the product of the *brown* locus appears to be a tyrosinase of low specific activity which stabilizes the other two enzymes. These studies reveal the complexity of the control of pigment formation, which is an essential protectant against the carcinogenic effect of UV radiation. Vincent Hearing has also shown that the melanoma surface antigen B700 is a critical determinant of the immune response to melanoma in the mouse. Monoclonal antibodies to this antigen can be used to treat experimental melanoma pulmonary metastases.

Cathepsin L is a major cysteine acid protease which is secreted by many rodent and human tumor cells. Michael Gottesman's group has shown that human renal cancers, testicular cancers, and non-small cell lung cancers have especially high levels of cathepsin L mRNA, indicating that it may be a useful biological marker for the presence of these cancers, and a potential site for anti-cancer therapy. Regulation of the mouse cathepsin L gene is complex, with regulatory elements present both "upstream" and "downstream" from the start site of transcription.

The Mechanism of Energy-Dependent Proteolysis

Michael Maurizi and his co-workers have begun to elucidate the mechanism of energy utilization by bacterial proteases. One such protease, Clp, consists of two components. ClpA is a regulatory component which is assembled into a hexamer in the presence of ATP; hydrolysis of ATP is not required for this reaction. This ClpA hexamer interacts with the dodecameric catalytic subunit, ClpP, to form an active enzyme. This complex formation is reminiscent of the assembly of "proteasomes" into the ATP-dependent 26S ubiquitin-conjugate degrading protease observed in mammalian cells. ATP is also required in a second step to allow proteolysis by the ClpA:ClpP complex. The determinants of specificity of the Clp protease are not known. However, studies using LacZ-fusion proteins have indicated that the determinants of specificity *in vivo* and *in vitro* may be different, suggesting the involvement of other host factors in

the proteolytic recognition process. Another approach to the problem of proteolytic specificity has been to isolate mutants of ClpP with altered autoprocessing sites.

The Mechanism of Antigen Processing

Research in the Chemistry Section under the direction of Ettore Appella has emphasized the use of techniques in peptide and nucleic acid chemistry to study the problem of antigen presentation by class II MHC molecules. The immunodominant portion of hen egg lysozyme (residues 107-116) has been compared to other peptides which interact with I-E^d and found to contain multiple basic amino acids. In contrast, peptides which bind to I-E^k have two hydrophobic amino acids followed within 6-8 residues by a lysine. A novel technique for isolation of pure exogenous/I-E^d complexes free of self-peptide/I-E^d complexes has been developed. In addition, micro-HPLC techniques have been utilized to purify three distinct peptides with the goal of understanding the nature of self-peptides and why self-peptides do not saturate class II MHC molecules. Studies on stability and recycling of class II MHC molecules have shown that these molecules have a long half-life and continually recycle from the cell surface into the cell, suggesting a mechanism whereby peptide exchange could occur.

Additional studies in the Chemistry Section have highlighted the role of phosphorylation of the gamma interferon receptor in mediating its effects on the intracellular signalling pathway, and have extended studies on two different tumor specific transplantation antigens. One of these antigens, found on methylcholanthrene-induced mouse sarcomas, consists of 84 kDa and 86 kDa proteins which are the mouse equivalent of the 90 kDa heat shock proteins, possibly complexed with putative antigenic peptides. The second antigen is a novel 110 kDa glycoprotein whose biochemical and immunological characterization is in progress. Finally, peptides have been synthesized which mimic the binding of the cellular EPl transcription factor to regulatory sequences in HIV LTRs. These studies represent an initial effort to understand the structural and mechanistic features of regulation of HIV transcription and to design strategies to interfere with HIV expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 03229-21 LCBGY
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) T-Cell Antigen Recognition and Tumor Antigens		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E. Appella	Medical Officer LCB, NCI
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	K. Sakaguchi	Visiting Fellow LCB, NCI
	S.J. Ullrich	Senior Staff Fellow LCB, NCI
	M. Fiscella	Guest Researcher LCB, NCI
	E. Leonard	Medical Officer BRMP, NCI
	K. Ozato	Research Microbiologist LDMI, NICHD
COOPERATING UNITS (if any) Cytel, La Jolla, CA; Preclinical Research, Sandoz Ltd., Basel, Switzerland; Mikrobiologisk Institute, Copenhagen, Denmark		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Chemistry		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither B <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Our laboratory is investigating the structural and functional relationship between peptide antigens and class II major histocompatibility (MHC) molecules. The model of interaction between the lysozyme peptide 107-116 and the I-E^d and I-E^k molecules has indicated that this peptide assumes an extended configuration in the binding site of the MHC molecule. Two different regions of the lysozyme peptide 107-116 which interact with I-E^d and I-E^k MHC molecules have been identified. Analysis of these and other peptides has revealed two distinct sequence "motifs" for peptides which bind to I-E^d and I-E^k molecules. For peptides that bind to I-E^d, multiple basic amino acid residues are essential, whereas peptides that bind to I-E^k, two hydrophobic amino acids followed 6-8 residues apart by a basic residue are required. A naturally processed peptide derived from lysozyme as well as three self peptides have been identified as bound to purified class II molecules. Highly purified peptide/MHC complexes have been obtained which stimulate a hybridoma specific for I-E^d and the lysozyme peptide 107-116. However, these complexes are devoid of stimulatory activity for three I-E^d alloreactive hybridomas. Subcellular site for peptide binding to class II molecules appears to be in a post-endocytic compartment. Our recent data indicated that <i>de novo</i> synthesized class II molecules are responsible for the binding of exogenous protein antigens, whereas peptide antigens bind to pre-existing mature class II molecules via an exchange mechanism.</p> <p>The structure of the γ-interferon receptor and its pathway of signalling, as well as the nuclear binding factors involved in the regulation of expression of class I and class II molecules and their relationship to the transcription of the HIV-virus are being pursued. The immunological significance of tumor antigens is a major problem in tumor biology. Recently, we have purified a glycoprotein (gp110) from murine sarcomas which has tumor rejecting activity restricted to the tumor of its origin. The analysis of the gp110 is well on its way to defining this antigen in both <i>in vivo</i> and <i>in vitro</i> immunological assays.</p>		

Major Findings:(1) T-Cell Antigen Recognition and Major Histocompatibility Complex (MHC) Antigens

T-Cells recognize antigens on the surface of antigen-presenting cells (APC), but only after the antigen has been processed by an APC and then displayed in association with MHC molecules on the APC surface. We have chosen to analyze the details of antigen presentation of hen egg lysozyme (HEL), as we previously demonstrated a strong T-cell response to HEL in BALB/c mice. One region of HEL, HEL 107-116, was found to be immunodominant, eliciting a strong T-cell response, when bound to the murine class II I-E^d molecules. Subsequent to these studies, seven other peptides from other proteins were also found to bind strongly to I-E^d. When the core regions of these peptides were compared to the core region defined for HEL 107-116, a "motif" containing multiple basic residues was defined. Recently, we have analyzed the interaction of the HEL 107-116 peptide with the murine class II I-E^k molecules. A difference was found in the MHC contact residues between the HEL sequence 107-116 and the I-E^d and I-E^k molecules, suggesting that the HEL peptide assumes a different conformation in the binding site to these two MHC molecules. A model of the interaction between HEL 107-116 peptide and the I-E^k molecules has been derived, by combining the experimental data with energy minimization procedures. The HEL 107-116 peptide appears to assume an extended configuration in the binding site of the MHC class II molecule. Two subsites within the HEL peptide are involved in the interaction with I-E^k and I-E^d molecules. The first subsite, K 116 is shared; the second subsite is not shared and involves the hydrophobic residues V 109 and V 110 for the I-E^k molecules, whereas peptide residues R 112 and R 114 are critical for the interaction with the I-E^d molecules. We have further examined the sequences of several peptides which bind the I-E^k molecules and a "motif" can be derived characterized by a K preceded, six to eight residue positions before, by two hydrophobic amino acids. This "motif" is useful for predicting the binding of antigenic peptides to the I-E^k molecules. Recent studies have shown that I-E^k class II MHC molecules can be expressed in a glycan-phosphatidyl inositol (GPI)-anchored form and can be removed in a soluble form from the cell surface with a phosphatidyl-inositol-specific phospholipase C. With these molecules we have initiated a study of their interaction with peptides and with the cognate T-cell receptor molecules under detergent-free conditions. A wide range of physical studies are being carried out to better define the interaction of class II MHC molecules with various peptides.

The function of class II molecules is to act as a cellular receptor for peptide antigens and present them to T-cells. However, one important question is how these class II molecules avoid saturation from self peptides. It is conceivable that in order to maximize the effectiveness of presentation to T-cells of foreign antigens, it would be advantageous for the organism to minimize the number of self binding determinants contained in its own proteins. We have analyzed the binding to the MHC class II molecules as well as the immunogenicity of peptides encompassing the entire sequence of mouse and human β 2 microglobulin. No differences have been found in the binding to either mouse or human class II molecules of all the peptides of the mouse and human β 2 microglobulin. These data suggest that the MHC class II molecules bind indiscriminately peptides corresponding to either self or non-self proteins. Thus the question of how class II molecules avoid being saturated by self peptides remains open. Clarification of the processing and presentation

pathways of endogenous and exogenous antigens, within APCs, may help to define how self/non-self competition might influence T-cell activation. To reach the above goal, the nature of physiologically-produced peptide antigens is being examined by using material eluted from purified MHC molecules obtained from HEL-pulsed B cells. We have devised a protocol for isolating and characterizing these peptides. Purified I-E^d molecules were treated with 2 M acetic acid and the acid eluate subjected to reverse phase HPLC chromatography. Analysis by high sensitivity mass spectrometry has indicated a significant heterogeneity of both self and naturally processed HEL peptides. At this time, we have obtained partial sequences from three chromatographically distinct peaks and these are not peptides derived from HEL. Antibodies to these peptides are being made to determine first the nature of these self peptides; and second whether or not I-E^d positive animals are tolerant to these peptides. Characterization of these peptides will open new avenues for studies on self antigen processing, presentation and tolerance.

Several reports have shown that alloreactive T-cells show a fine specificity for MHC molecules and it has been demonstrated that residues of the MHC molecules constitute at least part of the ligand to which alloreactive T-cell receptors bind. The X-ray crystal structure of HLA-A2 protein has provided evidence that MHC-bound self peptides might contribute to such recognition. We have used T-cell hybridomas, alloreactive for I-E^d to determine whether alloreactive T-cells are specific for the self-peptide/MHC complex or the MHC molecule alone. The experimental approach is to determine if a highly purified HEL-107-116/I-E^d complex is reactive with alloreactive hybridomas to the I-E^d molecules. We have designated a two-step affinity chromatography procedure to isolate class II MHC preparations fully occupied by the HEL peptide 107-116. For the purification of the complex we have attached at the N-terminal of the peptide HEL 107-116 a long chain thiol-cleavable biotinylated reagent, thus allowing specific retention of peptide/MHC complexes to an avidin column. The peptide/MHC complexes were subsequently recovered from the column by elution with reducing agents such as 2-mercaptoethanol. As expected, the pure complexes were highly stimulatory for an I-E^d-restricted and HEL 107-116 specific hybridoma. However, in striking contrast, at the highest concentrations tested they were completely devoid of stimulatory activity for three I-E^d alloreactive hybridomas. These results argue for a particular allo-self peptide requirement in class II MHC allorecognition.

Antigens presented by antigen-presenting cells (APC) are proteolytically processed to peptides. These peptides in turn are bound by the antigen-restricting element either a class I or class II MHC molecule. Peptide-binding to class I molecules usually occurs in the E.R. In contrast, the exact subcellular site for peptide binding to class II molecules is not certain although it appears to be in a post-endocytic compartment. Previously, we determined that when APC are incubated with either cycloheximide or Brefeldin A, the ability of APC to present exogenous protein-antigen was abrogated. However, these inhibitors did not prevent APC from binding and presenting peptides corresponding to the immunogenic region of the whole protein antigen. These data indicated that de novo synthesized class II molecules are the major class II species responsible for the binding of exogenous protein antigens after intracellular antigen processing, whereas peptide antigens are apparently able to bind to pre-existing mature class II molecules via a peptide exchange mechanism. Several features of peptide exchange suggested that it could occur during recycling of cell surface class II molecules via endosomes. Several lysomotropic amines have no effect but methylamine, a reported inhibitor of

endocytosis, did inhibit peptide exchange. Our biochemical analyses of class II molecules have revealed that the majority reach the cell surface ~3hr after synthesis. The turnover of the class II molecules was found to be biphasic; one pool had a 5hr half life, the other 196hr. Thus mature class II molecules have an extremely long half life. Furthermore, the steady state level of class II molecules was unaffected by 18hr cycloheximide treatment. Preliminary experiments indicate that class II molecules are endocytosed and recycled back to the surface in cycloheximide treated cells. Altogether, our data indicate that class II molecules continually recycle. Thus, peptide exchange could conceivably occur during class II molecule recycling. Experiments are underway to examine the effect of inhibitors of peptide exchange (e.g., methylamine) on class II molecule recycling.

Interferon gamma (IFN- γ), a cytokine produced by activated T-lymphocytes, is a major regulator of specific immune responses. The first step of IFN- γ action on target cells is the binding to a specific cell-surface receptor. The mouse and human IFN- γ receptors have been cloned and their structure reveals a typical transmembrane protein with an extracellular and an intracellular portion. One striking observation is that both the mouse and human receptors are rich in serine residues and these serine residues are highly conserved in the intracellular region. This observation has stimulated the analysis of the degree of phosphorylation of the receptor as a consequence of ligand binding. We have found that the mouse IFN- γ receptors are phosphorylated in response to the ligand in a specific and dose dependent fashion. The same results have been obtained by others for the human IFN- γ receptors. The precise function of receptor phosphorylation is not known, but hopefully will provide a way to identify a specific IFN- γ responsive kinase activity. Previous studies have indicated that formation of a IFN- γ receptor capable of inducing cellular responses requires two species specific components, the receptor and a second protein that has not yet been identified. In order to identify this protein, we are screening human cells transfected with an expression vector library carrying the murine specific signalling pathway component. No positive clones have so far been identified. IFN- γ induce the transcription of MHC class I genes via the interaction of DNA binding factors with a conserved IFN consensus sequence (ICS) located upstream of the class I gene promoter. We have obtained the structure of one ICS binding protein which binds an IFN response motif of about 10bp which is shared by many IFN-regulated genes. The first 115 amino acids share 45% sequence homology with two other factors, IRF-1 and IRF-2, which bind the same or very similar DNA sequences. We have synthesized the first 76 amino acids of the above factor, in order to ascertain its DNA binding capability. We have also raised antibodies to it and experiments are underway to determine whether the cloned factor and the naturally occurring ICS-binding proteins are the same or are products which undergo post-translational modifications, and then move to the nucleus to activate the transcription of specific sets of genes.

The MHC class II genes encode a set of heterodimeric cell surface glycoproteins responsible for the presentation of processed antigens. The transcription of these genes is tightly controlled on several levels and aberrant regulation is associated with certain disease states in humans. We have recently initiated a study on the regulation of MHC class II gene expression in transgenic mice. Several strains of transgenic mice have been produced carrying the murine or the human class II gene E α or DR α genes. The mouse receiving the gene of interest has invariably been a C57BL/6 or SJL or a hybrid of the two, since these animals cannot express their own E α gene due to a 600 bp deletion that encompasses the promoter region and the first exon. We

have obtained transgenic mice that express the human class II MHC molecule DR α on a genetic background in which the equivalent endogenous gene, EA' is not expressed. Our recent data as well as published observations show that despite amino acid differences at 25% of the 230 residues comprising DR α and EA, as well as divergences in flanking regulatory sequences, the DR α polypeptide pairs efficiently with the mouse E β and quantitatively restores its expression at the cell surface. In addition, the tissue distribution of transgenic DR α products closely resembles the distribution of their murine homolog, the endogenous EA product. These findings demonstrate a high degree of conservation between these MHC genes of two evolutionary different species and suggest that the shared phenotype is controlled by discrete conserved regions of the EA and DR α molecules. The ability of the transgenic mice to present I-E restricted antigens is in progress. It remains to be tested, however, if this interspecies conservation also apply to the more polymorphic members of the two MHC gene families.

(2) Tumor Specific Transplantation Antigens

Tumors induced by chemical carcinogens express tumor specific transplantation antigens (TSTAs). The immune response to these antigens is, for the most part, limited to a cellular immune response. We have purified from a 3-methylcholanthrene-induced mouse fibrosarcoma, a TSTA consisting of two closely related proteins of 84 and 86 kDa. These two proteins were identified as the murine equivalent of the 90 kDa heat shock proteins. However, recently a Mono Q purified preparation could be subdivided into an immunogenic and non-immunogenic fraction. The non-immunogenic pool was found to be enriched for a multicatalytic protease or proteasome. This finding suggests that an important function of the HSP90 heat shock proteins may be the binding of the proteasome. The immunogenic pool contained essentially pure HSP90 protein. If the actual TSTA activity represents a copurified component other than HSP90, then it must be present at the concentration of less than 0.1%. To resolve this problem we are raising specific CTL cell lines from peritoneal exudate cells of mice hyperimmunized with the pool showing a TSTA activity. If the CTL cells were shown to proliferate in response to the purified fractions, then they can be used as a method highly sensitive to detect any copurified protein or peptides eliciting the TSTA activity.

Recently, in collaboration with Dr. DeLeo of the Pittsburgh Cancer Institute, we have isolated and identified a glycoprotein of 110,000 kDa, from antigenically unrelated murine sarcomas, including Meth A sarcoma, which has tumor rejection-inducing and anti-tumor CTL-inducing activities restricted to the tumor of its origin. The N-terminal peptide sequence of purified Meth A gp110 was determined and shown to be distinct from any previously described protein. Monoclonal antibodies (mAb) of rat origin have been generated against Meth A gp110. These antibodies recognize gp110 in several murine tumors. Sera of tumor-bearing mice have also been shown to contain antibodies which detect gp110. The rat anti-gp110 mAb also detect a cross-reacting gp110 moiety in cell-free extracts of several human tumors tested, indicating its potential usefulness in the analysis of gp110 in human cancer. Anti-tumor specific CTL cell lines have been cloned from peritoneal exudate cells (PEC) of mice hyperimmunized with the antigenically unrelated BALB/c sarcomas, Meth A, CMS4 and CMS5. An anti-Meth A CTL line, designated CTLMA-9C, has been cloned and its cytotoxic reactivity and proliferative responses shown to be restricted to Meth A sarcoma. Growth of Meth A but not CMS5 sarcoma was significantly inhibited in mice immunized with irradiated CTLMA-9C cells, suggesting that a determinant recognized by CTLMA-9C is functional in tumor rejection assays.

Finally, CTLMA-9C cells were shown to proliferate in response to purified gp110. The analysis of Meth A gp110 is well on its way to defining this antigen in both *in vivo* and *in vitro*-based immunologic assay systems as a tumor rejection-inducing antigen. Future plans focus on the completion of the biochemical and immunological characterization of purified Meth A gp110, including analysis of gp110-peptide fragments for their amino acid sequence, which would facilitate the cloning of the gp110 gene, as well as the ability to stimulate the proliferative response of CTLMA-9C, which might identify the immunogenic portion of the molecule and site of polymorphisms.

(3) AIDS Research

It is known from clinical studies that the HIV virus exists in T-cells as a provirus integrated within the host genomic DNA. After exposure of the T-cells to various stimuli, HIV becomes transcriptionally active and re-enters the replicative cycle. The control of the initiation of transcription is mediated by cellular factors, such as EP-1, Nf-KB, which bind to nucleic acid sequences located in the LTR. An important initial step in developing a strategy for interfering with the binding of these factors to the HIV LTRs is the structural determination of the purified factor when complexed to the nucleotides which they bind. Peptides comprising a double and two single zinc finger regions of the HIV-EP1 binding protein have been synthesized by the solid phase method. Binding studies have shown that specific nucleotide-peptide complexes are formed by the double finger of the HIV-EP1 protein. The complexes formed by the double stranded nucleotides with the double finger peptide are being analyzed by three dimensional nuclear magnetic resonance spectroscopy. These studies represent an initial step in developing a strategy for interfering with the binding of transcriptional factors to the HIV LTRs and could lead to a rational design of antiviral drugs.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05597-02 LCBGY

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
Co-PI	S. Gottesman	Chief, Biochemical Genetics Section	LMB, NCI
Other:	M.W. Thompson	IRTA Fellow	LCB, NCI
	W. Clark	Chemist	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:

2.5

PROFESSIONAL:

2.1

OTHER:

0.4

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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)

ATP-dependent proteases are responsible for a major portion of the degradation of intracellular proteins in eukaryotic and prokaryotic cells, and play vital roles in cell division, adaptation, stress responses, and normal cell maintenance. *E. coli* has several ATP-dependent proteolytic systems. Work from this laboratory and others has shown that the *E. coli* Clp protease is representative of a family of ATP-dependent proteases found in all organisms and that these proteases are regulated by the heat shock response system. Proteases in this family are composed of two components, one a proteolytic enzyme and the other an ATP-dependent regulatory protein. Work during the past year has focused on the biochemistry of proteolysis by Clp protease and efforts to identify physiological targets of the enzyme. ATP has been shown to have two roles in activating Clp protease. ATP is required for the assembly of the complex between the regulatory component, ClpA, and the proteolytic component, ClpP. Association of ClpA and ClpP is promoted by non-hydrolyzable analogs of ATP, indicating an allosteric effect of ATP binding. Proteolytic activity of the activated complex requires ATP hydrolysis, indicating that ATP is involved in the catalytic cycle of peptide bond cleavage as well. Since ClpA and ATP are not needed for cleavage of short proteins or peptides, the ATP-hydrolysis-dependent step must involve alterations in the structure of large protein substrates or changes in the interactions between the protein substrate and the enzyme. The nature of the interactions between Clp and its substrates has been investigated using peptides that inhibit degradation of large and small substrates. Large substrates bind to Clp at both the active site and at a second site, since peptides have been found that inhibit protein cleavage but not peptide cleavage. The ClpA-independent peptide cleaving activity of ClpP may reflect the self-processing of ClpP *in vivo*. Mutational studies have begun to identify the amino acids involved in the specificity of cleavage of the precursor peptide from ClpP. A 19-amino acid peptide corresponding to the amino terminus of unprocessed ClpP is cut by ClpP *in vitro*, and further studies of binding of this peptide to ClpP and the kinetics of cleavage are under way.

Major Findings:

(1) 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. Molecular weight measurements indicate that one hexamer of ClpA interacts with a dodecamer of ClpP to form the active enzyme. Non-hydrolyzable analogs of ATP will allow complex formation but do not promote proteolysis. Thus, ATP is required in a second step by the active complex to stimulate protein degradation. An inactive mutant of ClpP with an altered active site serine has been purified and shown to interact with ClpA in an ATP-dependent manner similar to wild-type ClpP. Incubation of the mutant-ClpP/ClpA complex with protein substrate does not lead to protein degradation or to stimulation of the ATPase activity of ClpA, confirming that the increase in ATPase activity observed with the wild-type enzyme reflects a mechanistic coupling between the hydrolysis of ATP and peptide bond cleavage. Exchange reactions between mutant and wild-type ClpP in a complex with ClpA occur slowly under assay conditions, suggesting that the assembly of the active complex is not reversible under normal physiological conditions. The degradation of ClpA by ClpP observed *in vivo* and *in vitro* in the absence of available substrates may be a mechanism to deactivate Clp protease, which could then be reactivated by ATP-dependent assembly from newly synthesized ClpA. 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 mechanism of proteolysis of large proteins has been studied by measuring the kinetics of release of products during catalysis. Protein cleavage is largely processive with multiple peptide bonds cleaved within the same protein without release of partial degradation products. When partially inactivated ClpP was used, the average size of products released was larger, indicating that processive cleavage of a single protein may depend on the arrangement of active sites within the dodecameric protease. ATP is not needed for processive cleavage, since short proteins are cut in several sites without ClpA and ATP. Further studies using inhibitors of ATP binding and hydrolysis are being conducted to delineate the role of ATP in retaining the protein during the catalytic cycle or for facilitating the movement of protein substrates within the protease active site.

(3) Specificity of proteolysis by Clp protease has been investigated using both protein and peptides. Earlier several LacZ-fusion proteins were identified as substrates for Clp protease *in vivo*. These fusion proteins were purified and tested *in vitro* as substrates for purified Clp protease. Curiously none of the proteins was susceptible to degradation *in vitro*, suggesting that the proteins are altered in structure *in vitro* and not recognized by Clp protease. Confirmation of this explanation came from *in vivo* studies with *E. coli* defective in either Clp protease or "chaperonin" functions (DnaJ). A small amount of the RbsA-LacZ fusion protein accumulates in a stable form in wild-type cells, but in Clp mutants this stable form is found in 3-5 times higher amounts; it is this stable form of the protein that is purified. Achieving this stable conformation of RbsA-LacZ requires DnaJ, because in *dnaJ* mutants the fusion protein is completely degraded and no stable form accumulates.

Recently, Dr. Mark Thompson, a post-doctoral fellow working with me, has been investigating the specificity of Clp protease. Previous work indicated that several proteins inhibited protein degradation by Clp. Peptides derived from

partial digests of one of those proteins failed to inhibit Clp, suggesting that the intact protein interacts at more than one site on the enzyme. Dr. Thompson has also been surveying families of related peptides as inhibitors of either protease or peptidase activity. He has found several related peptides that show marked differences in inhibitory activity, and is planning to synthesize variant forms of the peptides to identify the sequence determinants for peptide binding to Clp protease. He has found several peptides that inhibit casein degradation but not peptide cleavage, consistent with the model that large proteins have several sites of interaction with Clp. We are currently investigating whether there is an allosteric site for protein substrates on Clp protease analogous to that observed with the ATP-dependent Lon protease.

(4) In collaboration with Dr. Susan Gottesman, we have been making site-directed mutants of ClpP altered in amino acids around the processing site. ClpP 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. They have found several mutants that are processed more slowly than wild-type ClpP and several appear to be processed at alternative sites. These latter mutants have lower proteolytic activity than the wild-type protease, suggesting that proper processing is required to allow ClpP to assume an active conformation. Since a purified unprocessed, mutant ClpP (ClpP-S111A) can adopt the native dodecameric structure and can interact with ClpA, 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. Dr. Satyendra Singh has cloned ClpP in a vector to allow synthesis of a mutant ClpP that differs from the processed enzyme only by having an amino terminal methionine. This protein is expressed at lower levels than wild-type ClpP and appears to have lower enzymatic activity, suggesting that a properly processed amino terminal end is required for full activity of ClpP.

(5) In collaboration with Dr. Valerie Stout, we have purified the RcsB protein, which is a positive regulator of capsular polysaccharide synthesis in *E. coli*. The RcsB protein will be used for DNA binding studies and for transcription assays to study regulation of expression of CPS genes. We will study the interaction between RcsB and RcsA, a highly unstable protein degraded *in vivo* by Lon protease. *In vivo* results indicate that interaction between RcsB and RcsA protects the latter from degradation. Attempts are underway to purify the RcsA protein, which is recovered from cells in inclusion bodies. Future plans are to use the purified Lon protease available in my laboratory to reconstruct the degradation of RcsA, to study the specificity of Lon protease and to determine the influence of interactions between components of a complex on the susceptibility to degradation.

Publications:

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

PROJECT NUMBER

Z01 CB 05598-02 LCBGY

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

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13.0

PROFESSIONAL:

13.0

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The simultaneous resistance of cancer cells to many different anti-cancer drugs is the major impediment to successful chemotherapy of metastatic disease. An important mechanism of multidrug resistance is expression of P-glycoprotein, a 170,000 dalton energy-dependent drug efflux pump which removes natural product drugs from the cell. We have continued our studies of multidrug resistance by an analysis of the mechanism by which this pump removes drug from within the plasma membrane or from the cytoplasm. In an *in vitro* vesicle system, ATP has been shown to be the preferred energy source, and many of the drugs which are transported compete with each other for a single site or small number of sites on the transporter. Labeling sites for the P-glycoprotein inhibitor ³H-azidopine occur in both the amino and carboxy-terminus of the protein, and these two sites appear likely to make up the single channel through which the drugs move. Molecular manipulations have identified the first intracytoplasmic loop as a domain involved in drug recognition, which is distinct from the drug labeling sites identified with ³H-azidopine. We have also developed an *MDR1* transgenic mouse whose bone marrow is protected from the cytotoxic effects of anti-cancer drugs by expression of P-glycoprotein. This model can be used to identify potent agents which inhibit the multidrug transporter *in vivo*, since these agents sensitize the transgenic mice to the leukopenia induced by chemotherapy. The *MDR1* cDNA can also be introduced into mouse bone marrow by retroviral infection. Such *MDR1* retroviral vectors should be useful for gene therapy to protect bone marrow during cancer therapy and to introduce non-selectable genes into bone marrow. New *in vitro* models of resistance to VP-16 and *cis*-platinum, not involving the multidrug transporter, are under development.

Other Professional Personnel:

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U. Germann	Visiting Fellow	LCB, NCI
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G.T. Merlino	Senior Staff Fellow	LMB, NCI
T. Manda	Special Volunteer	LMB, NCI
J. Campain	Special Volunteer	LMB, NCI

Major Findings:

(1) Cells selected for resistance to natural product chemotherapeutic drugs in culture often contain amplified copies of the *MDR1* gene. In human KB carcinoma cells, these amplified copies exist on episomes ranging in size from 700 kb to several thousand kb, which can be directly demonstrated after gamma irradiation on pulsed field gradient gels. The evolution of the episomes in one multidrug resistant cell line which had been selected in colchicine has been studied in detail. The amplified *MDR1* gene first appears on an episome of approximately 700 kb, at which time only "minute" chromosomes are visible cytogenetically. The next step in amplification involves a doubling in size of this episome, coincident with the appearance of "double minute" chromosomes. Selection to higher levels of drug resistance in this cell line results in alterations in size of the "double minute" chromosomes, and is consistent with the presence on the original episome of a gene other than the *MDR1* gene whose amplification is deleterious to the cell. Other multidrug resistant cell lines have different patterns of evolution of their extrachromosomal DNA, including multidrug resistant cells in which the primary event appears to be formation of an episome of several thousand kb.

(2) We have continued to characterize a model system in which energy dependent drug transport can be demonstrated in inside out vesicles derived from multidrug resistant cells. In this system, using ^3H -vinblastine as a primary substrate for transport into the vesicles, several other drugs handled by the transporter appear to compete for the transport site. These results suggest that there are only a small number, and possibly only one, transport site for the many different drugs which are pumped by P-glycoprotein. By testing a large number of nucleotide phosphates as potential sources of energy for the pump, we have shown that ATP is the preferred energy source. A three compartment kinetic model, which allows calculation of kinetic parameters associated with transport of drugs by the multidrug transporter across epithelial cell monolayers, has also been developed.

(3) The drug binding sites in P-glycoprotein have been localized using ^3H -azidopine as a ligand, various means of digesting P-glycoprotein into fragments and specific antibodies to identify the labeled fragments.

P-glycoprotein has been digested using trypsin, Staph V8 protease, and cyanogen bromide. Antibodies have been generated against most of the intracellular domains of P-glycoprotein using P-glycoprotein fragments synthesized in *E. coli* as antigens. To do this, cDNAs encoding P-glycoprotein fragments were linked to *Pseudomonas* toxin to produce highly immunogenic chimeric peptides. The immunoprecipitated ³H-azidopine-labeled P-glycoprotein fragments correspond to regions including the transmembrane domains of both the amino-terminus and the carboxy-terminus of P-glycoprotein. ³H-azidopine labeled can be inhibited by addition of vinblastine, which competes for the labeling sites. Both the amino-terminal and the carboxy-terminal labeling are equally inhibited by vinblastine, implying that both sites have equivalent affinity for both azidopine and vinblastine. These results suggest that the two labeled sites probably represent two halves of a single drug channel.

(4) By genetic manipulations, we have shown that the first cytoplasmic loop in P-glycoprotein contains determinants essential for drug recognition. During the selection of multidrug resistant cells in colchicine, high level resistance to colchicine resulted from substitution of a valine for glycine at position 185. Position 185 is in the first cytoplasmic loop of P-glycoprotein. We have made chimeras replacing this loop encoded by the *MDR1* gene with the homologous loop from the *MDR2* gene. These chimeras are non-functional with only 17 amino acids different out of a total of 89 in this region. Replacement of some of the *MDR2* amino acids in this region with the homologous amino acids from *MDR1* has resulted in a functional transporter, with some differences in substrate specificity. These results suggest that the first intracytoplasmic loop contains determinants for substrate specificity, despite the fact that the sites labeled by ³H-azidopine are not included within this loop.

(5) We have continued to develop the *MDR1* transgenic mouse as a model for testing agents which reverse drug resistance. In this model, mouse bone marrow, which expresses the human *MDR1* gene, becomes resistant to leukopenia induced by natural product anti-cancer drugs. Sensitivity to these drugs can be restored by agents, such as verapamil, quinidine, quinine, and cyclosporine A, which inhibit activity of the multidrug transporter. This transgenic mouse model enables the rapid screening of agents which can then be used in clinical trials to inhibit the multidrug transporter, thereby sensitizing drug resistant cancers to chemotherapy.

(6) It is possible to transfer multidrug resistance to cultured cells with a retroviral vector carrying the *MDR1* gene. We have shown that mouse bone marrow cells infected with this virus also become resistant to drugs such as colchicine and vinblastine, as detected in a GM-CFU assay *in vitro*. These infected cells can be introduced into mice where they form splenic foci which contain the *MDR1* gene. Since we have demonstrated that bone marrow expressing a transgenic *MDR1* gene can give a selective advantage to mice into which it has been transplanted, *MDR1*-retrovirus infected marrow should be selectable in mice. These studies form the basis for gene therapy of human cancer in which bone marrow can be protected from the cytotoxic effect of anti-cancer drugs, and in which the *MDR1* cDNA is used as a co-selected marker to introduce other genes into bone marrow.

(7) Many human cancers express the *MDR1* gene at levels comparable to those which give several-fold resistance *in vitro* and in our transgenic mice. Some human adult acute non-lymphocytic leukemias express such levels of

P-glycoprotein. When expression of P-glycoprotein is found, poor response to chemotherapy including daunorubicin and cytosine arabinoside is predicted. These results suggest either that expression of P-glycoprotein is itself responsible for poor response, presumably because the pump protects the cells from daunorubicin, or that expression of P-glycoprotein is a reporter for other changes in the cell that result in increased malignancy. Clinical trials using agents that reverse multidrug resistance will be needed to distinguish these two possibilities.

(8) Some tumor populations express very low levels of P-glycoprotein, and it has been uncertain whether this result reflects a small percentage of more positive cells, or a generally low level of expression in all cells. To distinguish these possibilities, we have developed a technique for the isolation of cells which express P-glycoprotein on their surfaces from mixed populations containing positive and negative cells. This technique employs ferromagnetic beads coated with antibodies to P-glycoprotein which allow the magnetic sorting of as little as 1% positive cells from a negative population. Preliminary results in which lymphoma cells from previously treated patients were analyzed, suggests that lymphoma samples from these treated patients have a mixed population of highly positive and negative cells. This magnetic sorting technique can also be used to isolate positive cells from a mixed population in order to study the mechanism of increased expression of P-glycoprotein.

(9) We have begun to isolate cell lines which are drug-resistant for reasons other than expression of P-glycoprotein. Three different cell lines are currently being characterized: (1) Human melanoma (FEM-X) cells selected for resistance to VP-16 in the presence of a tiapamil analog which inhibits the multidrug transporter; (2) Human hepatoma cells selected in *cis*-platinum. Prior to selection, human hepatoma lines show a multidrug resistant phenotype which is unrelated to expression of P-glycoprotein. Selection in *cis*-platinum should allow identification of novel mechanisms for intrinsic resistance in this important human cancer; (3) Human KB carcinoma cells selected for high levels of resistance in *cis*-platinum.

(10) *mdr* RNA levels in rodent cells, but not in human cells, can be increased by treatment with cytotoxic drugs. This increase in *mdr* RNA results in increased P-glycoprotein and increased drug resistance. There is an associated increase in transcription as measured by nuclear run-off, suggesting that increased *mdr* mRNA levels result from mRNA stabilization.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05599-01 LCBGY

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structural and Functional Characterization of the p53 Gene and Protein

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

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LAB/BRANCH

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SECTION

Chemistry

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS

2.5

PROFESSIONAL:

4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

The mechanism of the anti-proliferative effect of wild type p53 tumor suppressor gene was analyzed. We have found that cells expressing wild type p53 arrest in late G₁ stage of the cell cycle at or near the restriction point. Concomitant with this arrest the cells downregulate the expression of two proteins needed for entry into and completion of S phase, p34^{Cdc2} kinase and PCNA. Our analysis also indicated that the anti-proliferative effect of p53 correlates with an increased phosphorylation of the protein compared to mutant p53. Further, the sequence analysis of p53 from several human cell lines, derived from Gardner Syndrome patients, Raji B lymphoma, and lung cancer cells, were examined for mutant p53 expression. Although many human cancers have p53 mutations, no mutant p53 in the Gardner Syndrome fibroblasts nor in several of the lung cell lines were found despite elevated levels of p53. Thus, other mechanism(s) exist other than mutations which serve to stabilize p53 protein and downregulate its anti-proliferative activity.

Major Findings

p53 is a nuclear phosphoprotein of 393 amino acids which is overexpressed in many transformed cells. Mutant p53 has been associated with the oncogenic initiation and tumor progression of many human malignancies. Wild type p53 can have both a positive and negative effect on cell growth; being required to enter the cell cycle from G₀ and inhibiting cell growth when overexpressed. We and others have shown that wild type p53 has the ability to inhibit oncogenic transformation, behaving as a tumor suppressor gene or anti-oncogene. Missense mutations in p53 have been reported at over 40 locations in p53 in various human tumors. These mutations are restricted to the central region of the protein between amino acids 132 and 285. Various mutant forms of p53 have been found to behave as oncogenes by their ability to transform when cotransfected with the ras oncogene. Several mutational hot spots in p53 have been found and some show a strong correlation with a particular tumor type.

The ability of mutant p53 to inhibit the function of normal p53 appears to be through the formation of oligomeric complexes with wild type p53 present in the cell. Using mutant and wild type specific monoclonal antibodies, both mutant and wild type p53 in this complex appears to assume the conformation of mutant p53, resulting in the loss of function of the wild type protein. It appears that during the cell cycle wild type p53 can assume both a wild type and mutant-like conformation; the mutant conformation being associated with cell growth. The exact function(s) of p53 is not known although recent evidence indicates that it may serve to activate gene transcription. The goal of this research is to determine the specific post-translational mechanisms that controls p53 activity and to elucidate how wild type p53 exerts its antiproliferative effect.

Previously, our laboratory in collaboration with that of Dr. W.E. Mercer have shown that wild type p53 exerts an antiproliferative effect in both a human glioblastoma (T98G), and an hamster SV-40 transformed cell line (HR8). The T98G cell line was transfected with a plasmid construct in which expression of wild type p53 protein is under the dexamethasone inducible promoter, MMTV; a stable human cell line, GM47.23, was derived. We found that upon dexamethasone treatment cells entering the cell cycle from G₀ were prevented from proceeding into S phase. We now have found that the cells are arrested at the G₁/S boundary at or near the restriction point.

The T98G cell line also expresses an endogenous p53 protein, which we have determined has a mutation at codon 237. This allowed us to study the interaction between the wild type and mutant human p53 during and after growth arrest in the GM47.23 transfected cell line. We examined the conformation of wild type and mutant p53 in the GM47.23 cell line, their ability to cooligomerize, the half-life of the p53 protein and the relative degree of post translational phosphorylation. Previously, we reported that wild type human p53 is preferentially recognized by one p53 monoclonal antibody, Pab 1801, and poorly by the another monoclonal antibody, Pab 421, which binds better to endogenous mutant p53 protein. Using sequential immunoprecipitation it was determined that both wild type and mutant p53 are found in a complex, however in growth arrested cells significant levels of wild type p53 protein are found uncomplexed to endogenous mutant p53. Mutant/wild type p53 complex had a half-life of 4.5 h, whereas, the uncomplexed wild type p53 half-life was 1.9 h. Furthermore, use of a mutant-specific p53 Mab, 240, indicated that wild type p53 could exist in both a mutant (apparently when complexed with mutant p53) and wild type conformation (when not complexed with mutant p53). Finally, 2 dimensional gel analysis indicated that p53 exists as several different

phosphorylated species and that mutant p53 was less phosphorylated than wild type p53. Thus, our data suggests that the antiproliferative state of p53 may be controlled by phosphorylation at one or more sites. We are currently mapping the site(s) of differential phosphorylation of p53 by 2D phosphopeptide mapping. In parallel, we are using site directed mutagenesis to destroy the known phosphorylation sites of p53 and determine their effect on the antiproliferative properties of wt p53.

In order to study the mechanism whereby wild type p53 exerts its anti proliferative effect we have examined the expression of certain genes involved in cell cycle progression, such as thymidine kinase, histone H1 and proliferating cell nuclear antigen, (PCNA, a subunit of DNA polymerase). We found that wild type p53 results in the inhibition of the expression of PCNA, at the protein and mRNA level, but no effect on thymidine kinase. As PCNA is essential for DNA synthesis, the cells are unable to start S phase. Inhibition of the cells in G₁ is also consistent with the fact that the S phase specific transcript, histone H1, is also not expressed. At present it is not known if p53 acts directly or indirectly on the expression of PCNA.

Recent reports have shown that the progression of the cell cycle through both M and G₁/S require the expression of the cell cycle regulated protein kinase, p34^{cdc2}. p34^{cdc2} activity is controlled via phosphorylation and by its association with another cell cycle regulated protein, cyclin. Moreover, mutant p53 is phosphorylated by p34^{cdc2} kinase in a cell cycle dependent manner. Therefore, we have examined the effect of wild type p53 expression on p34^{cdc2} expression in cells entering the cell cycle from G₀. p34^{cdc2} kinase activity was inhibited 5 fold upon wild type p53 expression. These changes in p34^{cdc2} activity were accompanied by a shift to nonphosphorylated form of the protein. Currently, we are examining if the reduced phosphorylation is due to changes in the activity of a p34^{cdc2} specific kinase and/or phosphatase. Exponentially growing cultures were also found to have reduced p34^{cdc2} kinase activity. Thus, wild type p53 expression correlates with the loss of active/functional p34^{cdc2} which is consistent with the notion that growth arrest by p53 may be due to the loss of active p34^{cdc2} kinase.

p53 mutations have been found in many human malignancies, however, human B cell tumors have not been extensively analyzed for p53 mutations. Therefore, in collaboration with Dr. Pierre May, we have examined the biochemical properties and determined the nucleotide sequence the p53 found in the human Raji B cell lymphoma. The p53 protein expressed in these cells was found to contain double missense mutations at codons 213 and 234 on separate alleles. p53 in these cells displays a long half life and is found complexed to hsp70. Interestingly, a recent report indicates that another human tumor has the same double mutation in p53 and it is also a B cell tumor. These data could indicate that missense mutations in B cells may be restricted to a limited number of codons, as has been reported in the Li-Fraumeni syndrome. We plan to examine other B cell tumors to see if this is the case.

In collaboration with Dr. T. Lehman, p53 mutations in several human lung cell lines have been analyzed. Seven of the cell lines examined had elevated expression of p53 but only three were associated with a p53 mutation, whereas, in the other four no mutations were found, despite complete sequencing the p53 cDNA clones. Immunofluorescence analysis revealed that the expression of p53 in the non mutant cells was only found in 50% of the cells and restricted to the nucleus, similar to that observed in normal proliferating T lymphocytes. In contrast, all the nuclei of cells containing mutant p53 were positive for p53 staining. Finally, in collaboration with Dr. L. Kopelovich, we have been

examining the sequence of the p53 in cells from patients with Gardner syndrome (benign polyposis). These cells have previously been shown to express elevated levels of p53. Our analysis indicated that no mutations are present in these cells. Thus, the data from the lung cell lines and Gardner syndrome patients indicates that a mechanism exists to stabilize wild type p53 in both transformed and nontransformed cells, yet no anti-proliferative effect is observed. This stabilization may be due to a specific post translational change such as phosphorylation. Future studies will attempt to determine if the difference in p53 stability correlates with difference in p53 phosphorylation.

Publications:

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08705-15 LCBGY

PERIOD COVERED

October 1, 1990 to September 30, 1991

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: R. Fleischmann Staff Fellow LCB, NCI

D. Ray Research Biologist LCB, NCI

M. Gosse Guest Researcher LCB, NCI

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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

We are utilizing the Chinese hamster ovary (CHO) fibroblast as model to study the genetics and biochemistry of some aspects of the behavior of cultured cells. We are continuing to use this system to analyze the manner in which cyclic AMP regulates cell growth and gene expression in CHO 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. One such C subunit mutation has been characterized at the molecular level and shown to reduce mRNA for the α subunit. Defects in cAMP-dependent protein kinase block cAMP-stimulated transcription and reduce amounts of mRNA for the multidrug transporter in CHO cells. We have constructed several mutant forms of the CHO RI subunit and expressed these in *E. coli*. Bacterially expressed mutant RI's have defects in associating with the C subunit and in cAMP-stimulated dissociation of RI and C subunits. Another way to produce cells resistant to cAMP is by overproduction of cyclic nucleotide phosphodiesterase. A yeast phosphodiesterase (PDE) has also been expressed in CHO cells. Expression of this yeast PDE gene results in insensitivity to cAMP.

Major Findings:

(1) Some cAMP-dependent kinase mutants from CHO cells and Y1 cells are supersensitive to natural product cytotoxic drugs such as colchicine, vinblastine, adriamycin and puromycin. Revertant cells which have recovered normal levels of drug resistance have lost their protein kinase mutant phenotype. This result suggests that the expression of resistance to multiple drugs can be modulated by cAMP-dependent protein kinase, perhaps through a mechanism involving the multidrug transporter (*mdr1* gene product). CHO and Y-1 protein kinase mutants were found to have reduced levels of *mdr* RNA and reduced amounts of the transporter, indicating that in CHO cells, cAMP-dependent protein kinase modulates *mdr* mRNA levels. In confirmation of these observations, mutant Y-1 cells in which wild-type levels of protein kinase are expressed after transfection of C subunit vectors have increased *mdr* mRNA levels.

(2) We have continued to analyze mutants of cAMP dependent protein kinase in CHO cells. One such mutant, designated 10260, has reduced amounts of C subunit. Analysis with Chinese hamster specific $C\alpha$ probes shows a reduced amount of $C\alpha$ mRNA. PCR analysis shows that the residual mRNA has a reduced molecular weight, and that the deletion coincides with loss of an exon, probably owing to a defect in mRNA processing. This result suggests that $C\alpha$ is essential for normal cADepPK activity in CHO cells, but leaves uncertain the role of $C\beta$, which is also expressed in these cells.

(3) The full-length cDNA for the Chinese hamster RI subunit and the $C\alpha$ and $C\beta$ subunits have been expressed in *E. coli*. The recombinant RI protein is active as measured by its ability to inhibit C activity, but C subunit activity has not yet been demonstrated. Two novel RI mutants affecting the "hinge" region and a cAMP binding site have been tested for activity in this system. The cAMP binding mutant binds to C, but has an altered cAMP activation profile, suggesting that it may be a candidate dominant negative RI mutation for creation of a vector to inactivate cAMP dependent protein kinase. This and other dominant RI mutants (i.e., those which block activation of C in the presence of a wild-type RI subunit) will be introduced into a eukaryotic expression vector and used to inactivate cAMP-dependent protein kinase in cultured cells and, eventually, in specific tissues in transgenic mice.

(4) The yeast cyclic nucleotide phosphodiesterase (PDE) gene has been introduced into CHO cells where its expression ablates growth and transcription responses to cAMP. We have constructed five founder transgenic mice in which the yeast PDE gene under control of an insulin promoter has been inserted into the mouse genome. None of these have major defects in blood glucose levels, but one founder, when bred to homozygosity, has a pleomorphic syndrome of immunodeficiency and lymphoma which is under investigation.

(5) We have continued studies on cell surface properties of S49 mouse lymphoma cells and the relationship among cell adhesiveness, immunogenicity and tumorigenicity. Monoclonal antibodies to several novel antigens expressed on the surface of adhesive S49 variants have been prepared. These monoclonal antibodies will be used to define these surface antigens biochemically and functionally with respect to the increased immunogenicity and decreased tumorigenicity of these lymphoma cell variants.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08715-13 LCBGY
PERIOD COVERED October 1, 1990 to September 30, 1991		
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 D. Ray S. Chauhan	Research Biologist Research Biologist Visiting Fellow LCB, NCI LCB, NCI LCB, NCI
COOPERATING UNITS (if any)		
N. Popescu	Senior Investigator	Laboratory of Biology, DCE
LAB/BRANCH Laboratory of Cell Biology		
SECTION Molecular Cell Genetics		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p> Cultured mouse fibroblasts which are malignantly transformed or treated with TPA or growth factors such as PDGF synthesize and secrete the 39,000 M_r precursor to cathepsin L (also called MEP, for major excreted protein) in large amounts. Cathepsin L is a cysteine acid protease. Transformation, TPA and PDGF stimulate transcription of cathepsin L. The "promoter" region of the mouse cathepsin L gene has been isolated and shown to function in cooperation with downstream sequences to mediate TPA stimulation of cathepsin L promoter-CAT constructs. Cathepsin L is processed to 29 kDa and 20 kDa forms intracellularly in a reaction which appears to be, at least in part, dependent on active cathepsin L itself. A human procathepsin L cDNA has been cloned and sequenced. This cDNA recognizes at least two closely related, but distinct genes as determined by sequences obtained from cloned genomic segments and <i>in situ</i> hybridization to metaphase chromosomes 9 and 10. Analysis with cDNA probes specific to the form of human cathepsin L expressed in most tissues suggests that the functional gene maps to chromosome 9. Many human tumors express elevated amounts of mRNA for cathepsin L, with especially high levels in renal cancers, testicular cancers, and non-small cell lung cancers. </p>		

Major Findings:

(1) We have studied processing of cathepsin L by using specific inhibitors of this cysteine protease. Specific cathepsin L inhibitors block or delay the appearance of lower molecular weight (29 kda and 20 kda) forms of the protease in intracellular compartments. These results suggest that processing of cathepsin L may be autocatalytic.

(2) The upstream sequences of the mouse procathepsin L gene linked to CAT constructs are efficient promoter elements for CAT expression. However, these constructs are not regulated by TPA unless "downstream" sequences within the first 2 introns of the procathepsin L gene are added to the constructs. cAMP does stimulate catL-CAT constructs through "upstream" sequences alone. These results suggest that regulation of the mouse cathepsin L gene is complex, and involves interaction between "upstream" and "downstream" sequences.

(3) Genomic segments of the human cathepsin L gene have been obtained by isolated cosmids from a KB cell library using a human procathepsin L cDNA hybridization probe and by PCR using oligonucleotides derived from the known sequences of a human procathepsin L cDNA. Exonic genomic sequences derived from the cosmid diverge by about 10% from the known procathepsin L cDNA sequence suggesting that they represent a second closely related human procathepsin L gene or pseudogene. In contrast, a genomic segment derived by PCR contains exonic sequences identical to the cDNA.

(4) *In situ* hybridization of a procathepsin L cDNA to human metaphase chromosomes localizes to chromosome 9 and 10 (with N. Popescu). Using 5' non-coding sequences from the cDNA, localization exclusively to chromosome 9 can be demonstrated. Thus, there appear to be at least two procathepsin L homologs. The procathepsin L gene which encodes the mRNA found in most tissues is on chromosome 9.

(5) We are developing vectors to inactivate the mouse cathepsin L gene by homologous recombination in cultured somatic cells and embryonic stem (ES) cells with the eventual goal of producing mice lacking cathepsin L. Approximately 3 kb of mouse genomic DNA has been cloned on each side of a neo^R cassette. HSV-TK genes have been placed 3' and 5' to the cathepsin L sequences to allow negative selection in gancyclovir for non-homologous recombination events. When electroporated into mouse NIH 3T3 cells, this construct confers gancyclovir-sensitive G418 resistance, indicating that it is a functional "knock-out" vector.

(6) We have continued to measure cathepsin L mRNA in many normal human tissues and human cancers using a cloned human cathepsin L mRNA probe. All tissues express some cathepsin L mRNA, but higher levels are found in human kidney, liver, and lung. The highest levels of cathepsin L mRNA are found in human cancers such as renal adenocarcinomas, testicular cancers and non-small cell lung cancers.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09100-6 LCRGY

PERIOD COVERED

October 1, 1990 through September 30, 1991

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)

P.I.:	V. Hearing	Research Biologist	LCB/NCI
Other:	K. Tsukamoto	Visiting Fellow	LCB/NCI
	L. Law	Scientist Emeritus	LG/NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

2

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

B

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

This project is aimed at characterizing different parameters important to the growth and differentiation of melanocytes, and their significance to critical properties of the transformed melanocyte, termed malignant melanoma, especially with respect to its outgrowth and metastasis. These studies have identified, isolated and characterized three different melanogenic enzymes that regulate the quality and quantity of pigment produced by melanocytes. These proteins share significant homology and belong to a new family of tyrosinase-related proteins that are specifically expressed by mammalian melanocytes. In combination with a specific melanogenic inhibitor currently being characterized, mammalian melanogenesis is strictly regulated. Our studies have also continued the characterization of melanoma-specific antigens that are 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 melanoma 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.

Major Findings:

(1) Melanogenesis

We have continued our studies into regulatory factors important to the expression of melanocyte function, i.e., melanogenesis. Four different pigment related genes have been isolated 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 termini of those predicted proteins, and have raised specific antibodies against them. Those antibodies have been shown to recognize the expected proteins synthesized *in vivo* 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, which putatively maps to the *slaty* locus in mice, has yet another distinct catalytic function, the isomerization of DOPACHrome to dihydroxyindole-2-carboxylic acid (DHICA). Both of those enzymes have independent activities and there is no residual activity in either protein towards the other's substrates. TRP1, which maps to the *brown* locus in mice, has catalytic functions identical with tyrosinase, but with a specific activity only 5 to 10% that of tyrosinase. However, the major function of TRP1 appears to be through its interaction with the other two 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, enzymatic potential and stability, and thus their catalytic function. Another important regulator of mammalian melanogenesis is a low molecular weight inhibitor that functions by inhibiting the activities of the melanogenic enzymes noted above, 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.

(2) Melanoma Biology and Immunology

Our 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. 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 α -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.

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SUMMARY STATEMENT

ANNUAL REPORT LABORATORY OF CELLULAR ONCOLOGY DCBDC, NCI

October 1, 1990 through September 30, 1991

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 and the action of TGF- α on the epidermal growth factor receptor.

We analyzed chimeras between ras and rap-1A, which encodes a ras-like protein that can suppress ras-transformed cells. The results indicated that the respective effector regions of ras and rap-1A determined whether the protein induced cellular transformation or suppressed transformation, which suggests that rap-1A may suppress ras-induced transformation by interfering with the interaction between ras protein and its effector. Further analysis identified chimeras 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 p21rap-1A mediate sensitivity to each of the proteins with GAP activity, and ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap-1A, respectively, in NIH 3T3 cells.

In the studies with TGF- α , we have identified a system in which autocrine stimulation by TGF- α is more potent biologically than paracrine stimulation. Using a retroviral vector that encodes the full-length human TGF- α precursor protein, NIH 3T3 cells that expressed human TGF- α , which was processed and secreted normally, became morphologically transformed and were highly tumorigenic. By contrast to cells within the same petri dish that were not expressing TGF- α remained untransformed, as did cells that were treated with saturating concentrations of exogenous TGF- α .

Analysis of Papillomaviruses

Certain types of human papillomaviruses (HPVs) are frequently detected in human genital cancers, and therefore are designated high risk types, while other types are frequently detected in benign genital lesions, but rarely in carcinomas, and so have been designated low risk types. We have undertaken a comparative analysis of the gene products of high risk and low risk viruses in an attempt to gain insight into the important determinants of pathogenicity for the genital HPVs. The expression and activities of the E6 and E7 genes have been investigated because they are selectively retained and expressed in genital cancers. We have determined that the E6s and E7s of the two viral classes differ in their ability to induce cellular transformation and immortalization, with the apparent oncogenic potential of the viruses correlating with the *in vitro* activities of both E6 and E7. E6 and E7 also activate transcription of test promoters but there does not appear to be a

correlation between trans-activation and oncogenic potential. We have also determined that the E7s of high and low risk types differ in their ability to be expressed from polycistronic mRNAs.

The mechanisms of E6 and E7 induced transformation and immortalization are under investigation. For E6, we have determined the full-length protein product, but not the truncated E6* proteins unique to the high risk types, is required for these activities. Additional studies indicated that mutant p53 can functionally substitute for E6 (but not E7) in the immortalization of normal human keratinocytes but not in the transformation assay, suggesting that an interaction with wild type p53 may be important for immortalization but that a separable E6 function may be required for transformation. For E7, we are currently identifying the cellular proteins with which E7 interacts and evaluating the biological significance of these interactions.

Role of protein kinases in modulating cell growth and malignant transformation

The focus of this project is to better elucidate the role of altered transmembrane signaling in such processes as cell growth regulation, tumor promotion, cell differentiation, and cellular resistance to chemotherapeutic drugs. Protein kinase C (PKC) is increased significantly (5-7 fold) in the nuclei of drug resistant MCF-7 cells. A good correlation is found between the increased level of nuclear PKC and the increased degree of drug resistance. Results indicate that MCF-7/ADR cells, which are multidrug-resistant, contain a modified form of PKC alpha as determined by Western blot analysis, DEAE cellulose chromatography, and altered sensitivity to PKC inhibitors. These results suggest that elevated levels of a modified form of PKC alpha may act to modulate nuclear events to influence the development of multidrug resistance in MCF-7 cells.

A quantitative immunofiltration assay was developed to determine Raf-1 protein kinase (Raf-1 PK) activities in cell extracts. With this method two distinct forms of Raf-like enzyme activity were found to be present in the cytosolic fraction of NIH 3T3 cells. Immunohistochemical studies indicate that changes in cell population density and growth rate correlate with changes in the intracellular level and distribution of Raf-1 PK. Low levels of Raf-1 PK are found diffusely distributed throughout the cytoplasm in confluent, growth arrested cells, while the level of Raf-1 PK is increased in subconfluent, rapidly dividing cells and is found to be markedly elevated in the nuclear fraction. These results support the suggestion that Raf-1 PK may be involved in mitogenic signaling from the plasma membrane to the nucleus.

Evidence from two-dimensional gel analysis, Mono Q column separations, and specific immunoprecipitation studies indicate that the RII regulatory subunit, and to a lesser extent the RI regulatory subunit, of cyclic AMP-dependent protein kinases (PKA) may be covalently modified by retinoylation in HL-60 cells exposed to retinoic acid. The retinoylation of a subpopulation of RII, and perhaps RI, may play a role in the synergistic interrelationship noted between cyclic AMP and retinoic acid in regulating cell growth and differentiation.

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

PROJECT NUMBER
 Z01 CB 03663-15 LCO

PERIOD COVERED

October 1, 1990 through September 30, 1991

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)

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	N. Tayebi	Predoctoral IRTA Fellow	LCO NCI
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COOPERATING UNITS (if any)

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PROFESSIONAL:

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OTHER:

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- (a1) Minors
- (a2) Interviews

B

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

Oncogene studies have involved ras encoded proteins and the action of TGF- α on the epidermal growth factor receptor.

We analyzed chimeras between ras and rap-1A, which encodes a ras-like protein that can suppress ras-transformed cells. The results indicated that the respective effector regions of ras and rap-1A determined whether the protein induced cellular transformation or suppressed transformation, which suggests that rap-1A may suppress ras-induced transformation by interfering with the interaction between ras protein and its effector. Further analysis identified chimeras 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 p21rap-1A mediate sensitivity to each of the proteins with GAP activity, and ras-GAP and cytoplasmic rap-GAP are major negative regulators of p21ras and p21rap-1A, respectively, in NIH 3T3 cells.

In the studies with TGF- α , we have identified a system in which autocrine stimulation by TGF- α is more potent biologically than paracrine stimulation. Using a retroviral vector that encodes the full-length human TGF- α precursor protein, NIH 3T3 cells that expressed human TGF- α , which was processed and secreted normally, became morphologically transformed and were highly tumorigenic. By contrast to cells within the same petri dish that were not expressing TGF- α remained untransformed, as did cells that were treated with saturating concentrations of exogenous TGF- α .

Cooperating Units:

University of Alberta, Edmonton, Canada, Dr. J. Stone
 University Microbiology Institute, Copenhagen, Denmark, Drs. B. Willumsen and L. Beguinot
 Hebrew University of Jerusalem, Jerusalem, Israel, Dr. A. Levitzki
 Cetus Corporation, Emeryville, CA, Dr. F. McCormick

Major findings:

1. ras oncogenes. Normal ras proto-oncogene function is required for growth factor mediated mitogenesis, and mutationally activated ras genes have been identified in a variety of human and animal tumors. We have been studying ras function by examining proteins that influence the activity of ras protein and by performing structure-function analysis with mutants and chimeric genes. We have examined the influence of three proteins on ras: GAP, NF1, and rap-1A. 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 rap-1A gene encodes a ras-like protein that can suppress cells that are morphologically transformed by a highly oncogenic version of ras. rap-1A has its own GAP proteins (rap-GAP) that specifically accelerate its intrinsic GTPase activity.

To identify NF1 protein in mammalian cells, we raised antisera to the catalytic region of NF1. These antisera show the 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.

To study GAP, we examined the effects of GAP overexpression on transformation of NIH 3T3 cells by c-ras, v-ras, src, and mos. The transforming activity of src is dependent upon endogenous ras activity, while mos transformation is ras independent. Overexpression of GAP or its C-terminus (which contains its catalytic activity) inhibited transformation by c-ras or src, but did not impair transformation by v-ras or mos. The results suggest that GAP can act as a negative regulator in cells and that GAP overexpression can inhibit transformation by ras-dependent oncogenes. The data make it unlikely that GAP by itself is the ras target, but they are still consistent with the possibility that GAP may be part of a complex that forms the ras target; in that situation, GAP overexpression would affect the formation and/or activity of the complex.

A point mutation in the ras effector domain was shown to render c-ras and the mutationally activated v-ras temperature sensitive for transformation and to encode stable protein at the non-permissive temperature. In contrast to the biological activity, the sensitivity of the mutant c-ras protein to stimulation by GAP and NF1 was unimpaired at the non-permissive temperature. The dissociation between GTPase activity and the potential target function of GAP and NF1 demonstrates that a functional interaction between ras and GAP or NF1 is not sufficient for transmission of the ras signal.

We analyzed chimeras between ras and rap-1A to genetically localize the segments that mediate their opposite function. The results indicated that the respective effector regions of ras and rap-1A determined whether the protein induced cellular transformation or suppressed transformation, which suggests that rap-1A may suppress ras-induced transformation by interfering with the interaction between ras protein and its effector. Further analysis of the chimeric proteins identified chimeras 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, 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 p21rap-1A, respectively, in NIH 3T3 cells.

We also investigated the inhibition of cell growth by lovastatin, an antagonist of HMG CoA reductase which blocks the processing and membrane localization of ras proteins via inhibition of farnesylation. A series of NIH 3T3 cells transformed by oncogenes whose activity were dependent or independent of farnesylated ras were studied, including cells transformed by myristylated ras protein that is farnesylation independent. Treatment with lovastatin resulted in a time- and dose-dependent inhibition of cell growth in all lines tested. The inhibition did not show specificity for cells whose transformation is dependent upon farnesylated ras protein. It is therefore likely that the inhibition of other pathways affected by lovastatin, such as cholesterol biosynthesis or the processing of other cellular proteins, are responsible for the growth inhibition by lovastatin.

2. EGF receptors. A potential role for the normal EGF receptor in tumor formation has been proposed by others from the dual observation that increased numbers of apparently normal EGF receptors may be present in various human tumors and tumor cell lines and that many many tumors and cell lines overexpress TGF- α , which is a ligand for the EGF receptor. We have previously demonstrated that overexpression of normal human EGF receptors on NIH 3T3 cells renders them capable of tumorigenic transformation when the cells are treated with exogenous ligand (EGF or TGF- α). Using a retroviral vector that encodes the full-length human TGF- α precursor protein, we have now examined the effect of autocrine production by TGF- α on NIH 3T3 cells, which carry low numbers of endogenous mouse EGF receptors. Cells expressing the human TGF- α , which was processed and secreted normally, became morphologically transformed, in contrast to cells within the same petri dish that were not expressing TGF- α or cells that were treated with saturating concentrations of exogenous TGF- α , which remained untransformed. The TGF- α producing cells were highly tumorigenic in nude mice. The TGF- α effect appeared to be mediated by activation of the EGF receptor, since autocrine expression of TGF- α in NR-6 cells, which lack EGF receptors, did not have a phenotype, while autocrine TGF- α production of NR-6 cells that expressed human EGF receptors did induce cell transformation. The results suggest that in at least some instances autocrine stimulation by TGF- α can be more potent biologically than paracrine stimulation.

Publications:

Zhang K, Noda M, Vass WC, Papageorge AG, and Lowy DR. Identification of small clusters of divergent amino acids that mediate the opposing effects of *ras* and *Krev-1*. Science 1990;249:162-5.

- Zhang K, DeClue JE, Vass WC, Papageorge AG, McCormick F, and Lowy DR. Suppression of *c-ras* transformation by GTPase activating protein. *Nature* 1990;346:754-6.
- Collin C, Papageorge AG, Sakakibara M, Huddie PL, Lowy DR, and Alkon DL. Regulation of membrane excitability by *ras* oncogene proteins. *Biophys. J.* 1990;58:785-790.
- Collin C, Papageorge AG, Lowy DR, and Alkon DL. Early enhancement of calcium currents by *Ha-ras* oncoproteins injected into *Hermisenda* neurons. *Science* 1990;250:1743-5.
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- DeClue JE, Zhang K, Redford P, Vass WC, and Lowy DR. Suppression of *src* transformation by over-expression of full-length GAP or of the GAP C-terminus. *Mol. Cell. Biol.* 1991;11:2819-2825.
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01 CB 08905-10 LCO

PERIOD COVERED

October 1, 1990 through September 30, 1991

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
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	S. A. Lee	Biotechnology Fellow	LCO NCI
	J. W. Karaszkiwicz	Biotechnology Fellow	LCO NCI

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OTHER:

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 (a2) Interviews
 (b) Human tissues
 (c) Neither

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

The focus of this project is to better elucidate the role of altered transmembrane signaling in such processes as cell growth regulation, tumor promotion, cell differentiation, and cellular resistance to chemotherapeutic drugs. Protein kinase C (PKC) is increased significantly (5-7 fold) in the nuclei of drug resistant MCF-7 cells. A good correlation is found between the increased level of nuclear PKC and the increased degree of drug resistance. Results indicate that MCF-7/ADR cells, which are multidrug-resistant, contain a modified form of PKC alpha as determined by Western blot analysis, DEAE cellulose chromatography, and altered sensitivity to PKC inhibitors. These results suggest that elevated levels of a modified form of PKC alpha may act to modulate nuclear events to influence the development of multidrug resistance in MCF-7 cells.

A quantitative immunofiltration assay was developed to determine Raf-1 protein kinase (Raf-1 PK) activities in cell extracts. With this method two distinct forms of Raf-like enzyme activity were found to be present in the cytosolic fraction of NIH 3T3 cells. Immunohistochemical studies indicate that changes in cell population density and growth rate correlate with changes in the intracellular level and distribution of Raf-1 PK. Low levels of Raf-1 PK are found diffusely distributed throughout the cytoplasm in confluent, growth arrested cells, while the level of Raf-1 PK is increased in subconfluent, rapidly dividing cells and is found to be markedly elevated in the nuclear fraction. These results support the suggestion that Raf-1 PK may be involved in mitogenic signaling from the plasma membrane to the nucleus.

Evidence from two-dimensional gel analysis, Mono Q column separations, and specific immunoprecipitation studies indicate that the RII regulatory subunit, and to a lesser extent the RI regulatory subunit, of cyclic AMP-dependent protein kinases (PKA) may be covalently modified by retinoylation in HL-60 cells exposed to retinoic acid. The retinoylation of a subpopulation of RII, and perhaps RI, may play a role in the synergistic interrelationship noted between cyclic AMP and retinoic acid in regulating cell growth and differentiation.

Cooperating Units:

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LVC, NCI, NIH, Dr. P. Blumberg

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Lab. of Physiopath. Development, Paris, France, Drs. D.Evain-Brion, F. Raynaud

Dept. Radiation Med., Georgetown Univ. Sch. of Med., Washington, D.C., Dr. U. Kasid

Major Findings:

1. Protein Kinase C. Protein kinase C (PKC), plays a crucial role in the transduction of transmembrane signals generated in response to a variety of hormones and other extracellular stimuli, and also serves as the intracellular receptor for phorbol ester tumor promoter (TPA). Several lines of evidence indicate the possible involvement of PKC in modulating cellular resistance to antitumor drugs of the natural products class. Previous studies have demonstrated elevated levels of protein kinase C (PKC) activity in multidrug-resistant human breast carcinoma MCF-7/ADR cells compared to control drug-sensitive MCF-7/WT cells. Results of subcellular fractionation studies show that MCF-7/ADR cells have 5-8 times higher nuclear PKC activity than do control MCF-7 cells. There appears to be a good correlation between the level of nuclear PKC activity found and the degree of drug resistance in MCF-7 cells, as observed with two different MCF-7 cell lines. In both MCF-7 cell lines the most resistant cell types were found to contain the highest level of nuclear PKC activity, with less resistant cell types having intermediate levels of nuclear PKC activity relative to the very low level of PKC activity found in the control, drug-sensitive cell types. Western blot analysis of nuclei prepared from MCF-7/WT and MCF-7/ADR cells carried but with antisera specific for the different isoforms of PKC indicate that MCF-7/ADR cells contain markedly elevated levels of a slightly altered form of PKC alpha. Levels of PKC beta are similar in the two cell types, while PKC isoforms delta and zeta appear to be decreased in MCF-7/ADR cells relative to MCF-7/WT cells. Western blot analysis, CHAPS detergent solubilization, and DEAE cellulose chromatographic separation studies of nuclear PKC preparations isolated from MCF-7/WT and MCF-7/ADR cells all indicate both quantitative as well as qualitative differences between these two activities. These results suggest that elevated levels of a modified form of PKC alpha may play a role in modulating events to promote the development of multidrug resistance in MCF-7 cells.

2. Raf protein kinase. Another cytosolic protein kinase implicated in intracellular signal transduction is the Raf-1 protein kinase (Raf-1 PK), encoded by a member of the raf proto-oncogene family. Previously, we have shown that treatment of serum-deprived NIH 3T3 cells with the mitogen PDGF or with the phorbol ester tumor promoter TPA provokes a rapid redistribution of Raf-1 PK to the nucleus. Studies indicate that this nuclear Raf-1 PK is present in a catalytically active form that is tightly associated to the nuclear scaffold. Studies have been carried out to determine changes in the subcellular distribution of Raf-1 PK in normal and raf-transformed mouse fibroblast cell lines as a function of growth rate and cell population density. Raf-specific immunocytochemistry revealed that low levels of Raf protein are found localized in the cytosol of growth-arrested, high cell population density NIH 3T3 cells. In contrast, significant amounts of Raf-1 PK were found localized in the nuclei of growing cultures at intermediate or sparse population densities. Cells transformed by the v-raf oncogene were found to have high levels of v-raf truncated, active kinase present in the nuclear fraction regardless of cell population density or mitogen stimulation. These results suggest that Raf-1 PK may be a significant kinase in a cascade involved in the transmission of signal from the plasma membrane to the nucleus.

An immunofiltration assay was developed to specifically measure Raf-1 PK activity using a synthetic tetradeca peptide as substrate. With this assay it was determined that Raf-1 PK may exist in active and inactive forms within the cell. It was noted that two Raf-related protein fractions were eluted during Mono Q chromatographic separation of the cytosolic fraction of NIH 3T3 cells. The first peak of Raf protein (eluting at 150 mM NaCl) exhibited significantly greater Raf-1 PK activity than did the second peak of Raf protein (eluting at 450 mM NaCl), which appears to exist as a high molecular weight protein complex.

3. Retinoids in mediating cell growth and differentiation. The intent of this study is to elucidate the role and mechanism of action of retinoids in mediating cell growth and differentiation, and in acting as antagonists of tumor promotion. Previous studies from our laboratory have established that retinoic acid treatment of cells causes marked changes in cyclic AMP-dependent protein kinase (PKA) activity, and in the PKA regulatory subunits RI and RII. These results suggest an important synergy between retinoic acid and cyclic AMP to regulate cell growth and differentiation. Retinoylation (retinoic acid acylation) is a posttranslational modification of proteins shown to occur in a variety of eukaryotic cell lines. In collaboration with Dr. T. Breitman, we have determined by two-dimensional polyacrylamide gel electrophoresis, Mono Q anion exchange chromatography, and immunoprecipitation studies with antibodies specific to the RI and RII regulatory subunits of PKA, that the RII regulatory subunit can be covalently modified by retinoylation in HL60 cells exposed to tritiated-retinoic acid. Thus, an early event in retinoic acid-induced differentiation of HL-60 cells may be the retinoylation of subpopulations of both RI and RII.

Publications:

Olah Z, Komdy S, Nagashima N, Joo F, Rapp UR, Anderson WB. Cerebral ischemia induces transient intracellular redistribution and intranuclear translocation of the raf proto-oncogene product in hippocampal pyramidal cells. *Exp. Brain Res* 1991;84:403-10.

Gopalakrishna R, Anderson WB. Reversible oxidative activation and inactivation of protein kinase C by the mitogen/tumor promoter periodate. *Archives Biochem. Biophys.* 1991; 285:382-7.

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Evain-Brion, D., Raynaud, F., Tournier, S., Plet, A., and Anderson, W.B. Retinoic acid and cellular signal transduction. In: Saurat JH, ed. *Retinoids: ten years on.* 1991; in press.

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; in press.

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

PROJECT NUMBER
 Z01 CB 09052-03 LCO

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Analysis of Papillomaviruses

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

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	R. Kirnbauer	Special Volunteer	LCO NCI
	N. L. Hubbert	Microbiologist	LCO NCI
	J. V. Taub	Bio Lab Technician	LCO NCI

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 (a1) Minors
 (a2) Interviews

B

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

Certain types of human papillomaviruses (HPVs) are frequently detected in human genital cancers, and therefore are designated high risk types, while other types are frequently detected in benign genital lesions, but rarely in carcinomas, and so have been designated low risk types. We have undertaken a comparative analysis of the gene products of high risk and low risk viruses in an attempt to gain insight into the important determinants of pathogenicity for the genital HPV's. The expression and activities of the E6 and E7 genes have been investigated because they are selectively retained and expressed in genital cancers. We have determined that the E6s and E7s of the two viral classes differ in their ability to induce cellular transformation and immortalization, with the apparent oncogenic potential of the viruses correlating with the in vitro activities of both E6 and E7. E6 and E7 also activate transcription of test promoters but there does not appear to be a correlation between trans-activation and oncogenic potential. We have also determined that the E7s of high and low risk types differ in their ability to be expressed from polycistronic mRNAs.

The mechanisms of E6 and E7 induced transformation and immortalization are under investigation. For E6, we have determined the full-length protein product, but not the truncated E6* proteins unique to the high risk types, is required for these activities. Additional studies indicated that mutant p53 can functionally substitute for E6 (but not E7) in the immortalization of normal human keratinocytes but not in the transformation assay, suggesting that an interaction with wild type p53 may be important for immortalization but that a separable E6 function may be required for transformation. For E7, we are currently identifying the cellular proteins with which E7 interacts and evaluating the biological significance of these interactions.

In studies of Bovine papillomavirus (BPV) induced transformation of rodent fibroblasts, we have found that E5, the major transforming gene of BPV, can cooperate with a co-transfected *ras* gene in the induction of transformation.

Major Findings:

Background: Certain types of human papillomaviruses (HPVs) are frequently detected in human genital cancers, and therefore are designated high risk types, while other types are frequently detected in benign genital lesions, but rarely in carcinomas, and so have been designated low risk types. We have undertaken a comparative analysis of the gene products of high risk and low risk viruses in an attempt to gain insight into the important determinants of pathogenicity for the genital HPVs.

1. Comparative analysis E7. When expressed from isogenic constructions, the E7 from the low risk virus (HPV6) had detectable transforming activity, but it was less than that of the E7s from two high risk viruses (HPV16 and HPV18). The differences in transforming activities correlated with both the ability of the E7s to bind the Rb tumor suppressor protein and serve as a substrate for casein kinase II phosphorylation. The E7s of HPV16 and HPV18 were able to cooperate with either HPV16 E6 or HPV18 E6 to induce immortalization of normal human keratinocytes. In contrast, HPV6 E7 was unable to cooperate with HPV6 E6 or the E6s of the two high risk viruses. HPV6 E7 was not translated from a polycistronic mRNA that contained the upstream E6 open reading frame while the HPV16 and HPV18 E7s were. Splicing of the HPV16 E6E7 primary transcript, which in the high risk viruses allows translation of truncated E6* proteins, facilitated the translation of E7.

2. Comparative analysis of E6. We have determined that, when expressed from a strong heterologous promoter, HPV16 E6 can induce anchorage independent growth of NIH3T3 cells but HPV6 E6 can not. Similarly, HPV16 E6 can cooperate with the high risk E7s to induce immortalization of normal human keratinocytes but HPV6 E6 is inactive in the assay. Both high and low risk E6s can transactivate the adenovirus E2 promoter. These results suggest that trans-activation may not be a critical determinant of pathogenicity and may involve an E6 function that is separable from those required for immortalization and transformation. A unique feature of the genital high risk viruses is the potential of expressing truncated E6* proteins via splicing of the primary E6E7 transcript. It has been speculated that the E6* proteins may contribute to the greater biological activity of the high risk E6s. Therefore, we have compared the activities of mutant E6 genes that could express only the full-length HPV16 E6 or only the E6* proteins with the wild type gene that could express both. The clone expressing only the full-length protein was as active as the wild type clone in the three biological assay but the E6* clone was inactive and did not potentiate the activity of the full-length E6 expressing clone. Since E6* had no detectable biological activity, we speculate that it may simply be a by-product of the splicing reaction that has the primary function of regulating the expression of E6 and E7 in the high risk viruses.

3. Relationship between p53 and the activities of E6. It has been reported that the high risk E6 proteins, but not the low risk E6s, bind to the cellular p53 protein and induce its degradation in vitro. Since wild type p53 is believed to be a negative regulator of cell growth, it has been speculated that a similar interaction in vivo may be critical to the biological activities of E6. To test this hypothesis, we have examined the ability of mutant p53, which specifically inactivates wild type p53 via hetero-oligomer formation, to functionally substitute for E6 in the immortalization and transformation assays described above. Mutant p53 functionally substituted for E6, but not E7, in the immortalization assay. Coupled with our finding that transfection of wild type p53 inhibited proliferation of the keratinocytes, these results suggest that E6 could cooperate with E7 via inactivation of wild type p53. In contrast, mutant p53 was unable to transform NIH3T3 cells, even when expressed at high levels in the cells. Therefore, transformation of NIH3T3 cells by E6 may not involve a p53 dependent pathway.

4. Transformation of FR3T3 rat cells by BPV. Drug resistant clones of FR3T3 cells containing autonomously replicating BPV are often phenotypically normal initially but become morphologically transformed after passage in culture. This conversion occurred rapidly after co-transfection with the wild type virus but was delayed in mutants with interruptions in the E2 gene or a deletion of the late region. Manifestation of the transformed phenotype corresponded with an increase in viral early gene transcription. A frameshift mutation in the E5 gene abolished transformation but did not prevent autonomous replication.

5. Cooperation of BPV E5 and ras in the transformation of NIH3T3 cells. We have previously reported that E5 cooperates with co-transfected growth factor receptors, but not *c-src* or *c-fes*, to transform NIH3T3 cells. We have now determined that E5 can also cooperate with *c-ras^H* to induce focal transformation. We do not believe that E5 acts by affecting ras GTPase activity because cooperation was also seen with *v-ras^H*. These results reinforce the hypothesis that E5 acts by amplifying mitogenic signaling upstream of ras. In addition to contributing to our understanding of the mechanism of E5 transformation, the E5 cooperation assay has been useful in identifying transforming activity in certain *v-ras^H* mutants which by themselves were transformation defective.

Publications:

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Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1990 to September 30, 1991

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. Last year we reported that the VHL gene was isolated on the short arm of chromosome 3 in a 6-8 cM interval between RAF1 and D3S18. There are several new developments in our studies of von Hippel-Lindau disease.

As part of an attempt to locate the von Hippel-Lindau disease gene on chromosome 3, we evaluated 41 families with VHL. One large family was identified whose disease phenotype was distinct from typical VHL. The most common disease manifestation was pheochromocytoma. Few affected family members had symptomatic spinal or cerebellar hemangioblastoma; no affected family members had renal cell carcinoma or pancreatic cysts (common manifestations in typical patients with VHL). The manifestations in this atypical family were linked to RAF1 and to D3S18. These results suggest that there are mutant alleles or several contiguous genes (at least three) at the VHL locus associated with distinct tissue specificities.

We evaluated DNA-polymorphism analysis as a method for identifying disease gene carriers by prospectively comparing the results of RFLP analysis with a comprehensive clinical screening examination. Forty-eight asymptomatic individuals at risk of developing VHL were tested with probes located close to, and on either side of, the VHL gene. We found agreement between the results of the DNA test and the clinical screening examination in 41/42 individuals. Our conclusion was that DNA polymorphism analysis can identify individuals likely to carry the VHL disease gene among asymptomatic members of disease families.

A marker, D3S601, located between RAF1 and D3S18 was identified. This marker is the closest marker to VHL that we have identified. This marker is located between proximal and distal breakpoints in the region immediately surrounding the VHL gene.

We have made progress in studies of human small cell lung carcinoma and squamous cell carcinoma of the head and neck. In collaboration with Dr. Pamela Rabbitts, we have identified 43 probes that are homozygously deleted in a small cell lung carcinoma line. This small cell lung carcinoma line, U2020, is of interest because of previous studies which indicate the regions of the genome that are homozygously deleted usually contain tumor suppressor genes. We have shown the human squamous cell carcinoma of the head and neck is characterized by a loss of alleles at loci on chromosome 3p.

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 3p. Seventy one loci have been placed on a 3p map. For 28 loci, statistical analysis provided support for unique order. For 43 loci, statistical analysis did not provide support for a unique order, but provided information on regional location.

Immunopathology Section. During the past year, we continued work on three human host defense proteins--neutrophil attractant protein-1 [NAP-1], monocyte chemoattractant protein-1 [MCP-1] and macrophage stimulating protein [MSP]. Now that NAP-1 and MCP-1 have been sequenced and cloned, the major objective is to determine biological significance. Therefore, we developed reagents for measurement of these proteins in biological fluids, for immunohistochemical detection in tissue, and for identification by in situ hybridization of the producing cells.

New ELISAs for NAP-1 can distinguish between free NAP-1 and NAP-1 in an IgG immune complex. In a survey by sandwich ELISA of 6 human joint inflammatory fluids for NAP-1, we found a reactivity that was due to a NAP-1-IgG complex. There was no detectable free NAP-1. Since it was possible that proteins in the inflammatory exudate reflected concentrations in circulating blood, we looked for and found NAP-1-IgG, even in normal subjects. For a series of 27 normal subjects, the mean concentration of the complex was 82 ± 38 ng/ml. Individual values ranged from undetectable [less than 1 ng/ml] to 1000 ng/ml. Remarkably, in a series of 12 subjects, we found identical concentrations in serum samples from any one subject drawn at a 1 month interval. Thus, blood concentrations of the complex, though different in different individuals, appear to be finely regulated. The complex from the serum of a normal human subject was partially purified by applying serum to a monoclonal anti-NAP-1 column and eluting the complex with pH 2.5 glycine buffer. By HPLC gel filtration, the complex eluted at a position corresponding to that of an IgG marker, suggesting that one NAP-1 molecule was bound to one IgG. It is of great interest to find an immune complex in the circulation of normal individuals. [Free antibody to IL-1 in normal serum has been reported.] Although complex formation might be a mechanism for vascular clearance of NAP-1, it would be unusual for macrophages to recognize and ingest a complex comprising only 1 IgG.

MCP-1 can be produced by many different cells in response to various stimuli. We have put great effort into developing reagents for MCP-1, to study its biological role and possible contribution to disease. We have developed a sandwich ELISA for MCP-1, utilizing recently obtained monoclonal anti-MCP-1 and rabbit polyclonal anti-MCP-1. The ELISA has great sensitivity [detection limit of 30 pg/ELISA well], and does not detect related proteins in the host defense cytokine family. For studying animal models, Dr. Yoshimura has cloned MCP-1 cDNAs from several species, including rat, rabbit, guinea pig, and sheep. In contrast to MCP-1, it appears that NAP-1 may not occur in mice and rats. We used a NAP-1 oligonucleotide probe to screen a cDNA library constructed from Con A-stimulated rat spleen cells which were expected to express NAP-1 message. The probe was made from the portion where high similarity was expected. After washing under low stringency conditions we obtained more than 50 clones; none of them coded for NAP-1. By Northern blotting, human NAP-1 cDNA did not hybridize with mRNA extracted from Con A-stimulated rat spleen cells. Human NAP-1 cDNA hybridized to human, rabbit, and guinea pig genomic DNA digested by EcoRI, but not to mouse or rat

genomic DNA. [Human MCP-1 cDNA hybridized with genomic DNAs from all species.] Instead of NAP-1, rat and mouse may have a structurally related neutrophil chemoattractant. It could be GRO/KC which is a member of the same host defense cytokine family of proteins. Among small animals, the guinea pig would be good for *in vivo* experiments, since it has both MCP-1 and NAP-1.

After we succeeded in purifying MSP to homogeneity, our collaborators in the laboratory of Dr. Ettore Appella obtained partial sequences of endopeptidase digests of this 7- kD protein. Two of the fragments had highly significant sequence similarities to members of a protein family that include prothrombin, plasminogen, and hepatocyte growth factor [HGF]. The sequence similarities to portions of these proteins are of great interest. Prothrombin, plasminogen and the precursor form of HGF are all single chain proteins which can be activated by cleavage at one site to form α - and β -chains. For example, conversion of plasminogen to active plasmin occurs when a single Arg-Val bond is cleaved, resulting in a 2-chain structure held together by a disulfide bond. In addition to their sequence similarities, common features of MSP, HGF and plasmin include comparable molecular masses of the α - and β -chains, linkage of these chains by a single disulfide bond, and a valine residue at the N-terminus of each β -chain. Another structural motif shared by plasmin, HGF and prothrombin is a series of α -chain triple disulfide loops, called kringles. The kringle repeats twice in prothrombin, four times in HGF and five times in plasminogen. The sequence NYCARNPD, which forms the base of one of the disulfide loops, is identically conserved in plasmin, HGF and prothrombin, except for F instead of Y in prothrombin. Our finding of this motif in two fragments of the MSP α -chain strongly suggests that there are kringles in the α -chain, and provides added evidence for the relationship of MSP to this protein family.

The effect of MSP on resident peritoneal macrophage responsiveness to C5a led us to explore another receptor-mediated motility event that also requires an activating signal. Although C3b-coated erythrocytes [E1gMC3b] bind avidly to the C3b receptor of resident peritoneal macrophages, binding is not followed by phagocytosis unless an additional signal, such as a T-cell lymphokine or PMA, is provided, or unless cells are activated *in vivo* by induction of a peritoneal exudate. We confirmed that mouse resident peritoneal macrophages do not ingest E1gMC3b, and found that MSP stimulated ingestion. Whereas not more than 2% of resident macrophages ingested bound E1gMC3b, addition of MSP with the erythrocytes caused 50-60% of the macrophages to ingest E1gMC3b. Thus, MSP causes direct or indirect activation of both the C5a and C3b receptors of the resident macrophage.

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

PROJECT NUMBER

Z01 CB 08552-25 LIB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanism of Complement Fixation and Action

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

PI: T. Borsos Scientist Emeritus LIB NCI

COOPERATING UNITS (if any)

Department of Microbiology, University of Mainz

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21702

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

This is a long-range project investigating the mechanism of complement fixation and action. In particular, the interaction of antibody-antigen complexes with the first component of complement and the result of this interaction on the other components are investigated. The relation between antibody action and complement activation is also explored. Finally, the significance of complement in the humoral immune defense mechanism is studied.

Major Findings: Cultured human macrophages respond to certain stimuli and thereby are activated from resting state to active states. The activation process is gradual and involves several intermediate steps. We have correlated the activation steps as measured with biochemical markers with the appearance of cytotoxic property of the cell line U-937. Cytotoxic capability was measured by lysis of sheep red cells sensitized with rabbit IgG anti-Forssman antibody. U-937 cell stimulated with 12-O-tetradecanoyl-phorbol-13-acetate acquired characteristics of mononuclear phagocytes and the capacity to adhere to substrate. Adherence was essential for the appearance of cytotoxic activity: if adherence was prevented by culture conditions, cytotoxicity was not expressed.

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

PROJECT NUMBER

Z01 CB 08575-18 LIB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Inflammation

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

PI: E. Leonard Chief, Immunopathology Section LIB NCI

OTHER: T. Yoshimura Visiting Fellow
I. Sylvester Guest Researcher

COOPERATING UNITS (if any)

Ettore Appella Laboratory of Cell Biology, DCBDC

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21701

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

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.

Major Findings. We developed new ELISAs for neutrophil attractant protein-1 [NAP-1] that can distinguish between free NAP-1 and NAP-1 in an IgG immune complex. Serum from normal human subjects does not have detectable free NAP-1, but many sera have a NAP-1-IgG complex. For a series of 27 normal subjects, the mean concentration of the complex was 82 ± 38 ng/ml. Individual values ranged from undetectable [less than 1 ng/ml] to 1000 ng/ml. Remarkably, in a series of 12 subjects, we found identical concentrations in serum samples from any one subject drawn at a 1 month interval. Thus, blood concentrations of the complex, though different in different individuals, appear to be finely regulated. The complex from the serum of a normal human subject was partially purified by applying serum to a monoclonal anti-NAP-1 column and eluting the complex with pH 2.5 glycine buffer. By HPLC gel filtration, the complex eluted at a position corresponding to that of an IgG marker, suggesting that one NAP-1 molecule was bound to one IgG. To our knowledge, this is the first example of a circulating cytokine-immune complex in normal human subjects.

Monocyte chemoattractant protein-1 [MCP-1] can be produced by many different cells in response to various stimuli. We have put great effort into developing reagents for MCP-1, to study its biological role and possible contribution to disease. We have developed a sandwich ELISA for MCP-1, utilizing recently obtained monoclonal anti-MCP-1 and rabbit polyclonal anti-MCP-1. The ELISA has great sensitivity [detection limit of 30 pg/ELISA well], and does not detect related proteins in the host defense cytokine family. For studying animal models, Dr. Yoshimura has cloned MCP-1 cDNAs from several species, including rat, rabbit, guinea pig, and sheep. In contrast to MCP-1, it appears that NAP-1 may not occur in mice and rats. Instead of NAP-1, rat and mouse may have a structurally related neutrophil chemoattractant. It could be GRO/KC which is a member of the same host defense cytokine family of proteins. Our studies suggest that among small animals, the guinea pig would be good for in vivo experiments, since it has both MCP-1 and NAP-1.

After we succeeded in purifying macrophage stimulating protein [MSP] to homogeneity, our collaborators in the laboratory of Dr. Ettore Appella obtained partial sequences of endopeptidase digests of this 70 kD protein. Two of the fragments had highly significant sequence similarities to members of a protein family that include prothrombin, plasminogen, and hepatocyte growth factor [HGF]. A structural motif shared by plasmin, HGF and prothrombin is a series of α -chain triple disulfide loops, called kringles. The kringle repeats twice in prothrombin, four times in HGF and five times in plasminogen. The sequence NYCRNPD, which forms the base of one of the disulfide loops, is identically conserved in plasmin, HGF and prothrombin, except for F instead of Y in prothrombin. The presence of this motif in two fragments of the MSP α -chain strongly suggests that there are kringles in the α -chain, and provides added evidence for the relationship of MSP to this protein group.

Effect of MSP on resident peritoneal macrophage responsiveness to C5a led us to explore another receptor-mediated motility event that also requires an activating signal. Although C3b-coated erythrocytes [E1gMC3b] bind avidly to the C3b receptor of resident peritoneal macrophages, binding is not followed by phagocytosis unless an additional signal, such as a T-cell lymphokine or PMA,

is provided, or unless cells are activated in vivo by induction of a peritoneal exudate. We confirmed that mouse resident peritoneal macrophages do not ingest E1gMC3b, and found that MSP stimulated ingestion. Whereas not more than 2% of resident macrophages ingested bound E1gMC3b, addition of MSP with the erythrocytes caused 50-60% of the macrophages to ingest E1gMC3b. Thus, MSP causes direct or indirect activation of both the C5a and C3b receptors of the resident macrophage.

Publications:

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

PROJECT NUMBER

Z01 CB 08577-06 LIB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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, laboratory, and institute affiliation)

CO-PI: B. Zbar and M. Lerman

Other: F. Latif
M. Yao

Visiting Fellow
Nakasone Fellow

COOPERATING UNITS (if any)

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Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21702

TOTAL MAN-YEARS:

8.0

PROFESSIONAL:

4.0

OTHER:

4.0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects

(b) Human tissues

(c) Neither

(a1) Minors

(a2) Interviews

B

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

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 RAFI and D3S18. D3S601 is the closest marker we have identified to the VHL gene; it is located in a region immediately surrounding the VHL gene. We have isolated YAC clones for all markers close to the VHL locus and are in the process of cloning the region by YAC-based chromosome walking.

Forty-three probes homozygously deleted in a small cell lung carcinoma line were identified. Human squamous cell carcinomas of the head and neck were shown to be characterized by a loss of alleles at loci on chromosome 3p.

Major findings:

Von Hippel-Lindau disease

We evaluated DNA-polymorphism analysis as a method for identifying disease gene carriers by prospectively comparing the results of RFLP analysis with a comprehensive clinical screening examination. Forty-eight asymptomatic individuals at risk of developing von Hippel-Lineau disease were tested with probes located close to, and on either side of, the VHL gene. RFLP analysis predicted 9 disease gene carriers and 33 individuals with the wild-type (normal) allele among the 48 individuals at risk of developing von Hippel-Lindau disease. The tests were not informative in 6 individuals. All individuals predicted to carry the VHL gene had evidence of occult disease on clinical examination. There was no clinical evidence of von Hippel-Lindau disease in 32/33 individuals predicted to carry the wild-type allele.

As part of an attempt to locate the von Hippel-Lindau disease locus on chromosome 3, we evaluated 41 families with VHL from the United States and Canada. One large family was identified whose disease phenotype was distinct from typical VHL. The most common disease manifestation was pheochromocytoma. Few affected family members had symptomatic spinal or cerebellar hemangioblastoma; no affected family member had renal cell carcinoma or pancreatic cysts. These results suggest that there are mutant alleles at the VHL locus associated with distinct tissue specificities.

Small cell lung carcinoma and squamous cell carcinoma of the head and neck

In collaboration with Dr. Pamela Rabbitts, we have identified 43 chromosome 3p probes homozygously deleted in a small cell lung carcinoma line. Squamous cell carcinoma of the head and neck were characterized by a consistent loss of alleles at loci on 3p.

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

PROJECT NUMBER

Z01 CB 08578-02 LIB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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, laboratory, and institute affiliation)

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 MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

A genetic map has been constructed for the short arm of chromosome 3. This map extends from 3p26, the terminal band of 3p to the centromere. The length (sex-averaged) of this genetic map is 130 cM. A total of 71 loci were placed on the genetic map: 28 loci were assigned unique locations; 43 loci were assigned regional locations. The length of the male 3p map was 118 cM; the length of the female 3p map was 155 cM.

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1990 through September 30, 1991

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. Application of the basic understanding of these biological systems, serving as models for aspects of the cancer and other process, is accomplished through the use of advanced computing. Close collaborations provide valuable feedback and knowledge transfer between research domains. The Laboratory often develops computational and experimental methodology that is utilized by researchers in the biomedical community at large. Many of the theoretical studies have only been possible through the use of supercomputing facilities at the Advanced Scientific Computing Laboratory, FCRF.

Office of the Chief

Sequence Analyses in Virology, Cell and Molecular Biology. In the Office of the Chief, computerized analyses are used extensively 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 studies of sequences fulfilling analogous functions, such as promoters, taken from a variety of genes/organisms or by searches for overall sequence characteristics such as those required by genome packaging (Le, Currey, Konopka, Nussinov, Maizel and Owens).

New analytical tools for studies of proteins and nucleic acids have been developed and implemented (Barber, Le, Maizel, Nussinov, 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 Advanced Scientific Computing Laboratory. RNA structures up to 2000 bases in size have been predicted. Methods to assess the significance of predictions have used Monte Carlo simulations, evolutionary comparisons and biochemical data. New sequences were compared with computerized databases to detect relationships with known proteins.

RNA secondary structure methods have been refined to include alternate energy parameters, extended Monte Carlo simulations, and comparative studies to establish firmly the uncommon structural features in subregions of a number of sequences of HIV and other retroviruses (Le, Nussinov, Currey, Shapiro, 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 lookup procedure for predicting stability of random sequences, which accelerates surveys nearly 100-fold. Monte Carlo techniques are being developed that yield greater than 80% correct prediction of t-RNA structures, for more than 100 examined sequences.

Reasonable three-dimensional models for perforin, apo-lipoprotein and parts of HIV reverse transcriptase were built by computer methods. Biochemical data is testing these predicted models. This work is the beginning of what must become a more routine part of gene sequence analysis.

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 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, the why a wide variety of biomolecules 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 biomolecule 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 Glycosyl Transferases. Studies on the structure-function relationship of β 1-4galactosyltransferase and its modifier protein alpha-lactalbumin were continued (Qasba, Masibay and Boeggeman). cDNAs were manipulated and expressed both in mammalian and *E. coli* cells. The results show that the full-length 402 residue long galactosyltransferase that contains the amino-terminal membrane-anchoring domain when produced either in mammalian or *E. coli* cells is enzymatically active. The secreted form of the protein that lacks the first 70 residues, including the membrane-anchoring domain, is active only when produced in *E. coli* but not in mammalian cells. The galactosyltransferase enzyme without the amino-terminal membrane-anchoring domain when synthesized within the mammalian cells is either unstable or inactivated. The role of this domain may be to prevent the inactivation of the protein either by targeting it to the proper sites within the mammalian cells or by physically blocking the inactivation process.

The amino acid requirements for binding of Ca^{2+} in alpha-lactalbumin are being studied (Qasba and Kumar). Comparison of the sequences of Ca^{2+} -binding region of alpha-lactalbumin with the corresponding regions in the Ca^{2+} and non- Ca^{2+} c-type lysozymes suggest that in addition to the residues which have been identified by the X-ray crystal structure analysis as liganded to Ca^{2+} there are neighboring residues which also contribute to the binding of calcium ion.

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 ever 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. In a similar approach, studies have begun on 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).

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. 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, δ lysin, 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. Small peptide models have been built on the basis of 2D NOE NMR data indicating close atoms and molecular calculations. 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 question is whether or not the details of these conformations play a functional role in gene regulation; for DNA-protein interactions, these flexibilities and their asymmetries appear to play important roles. Models of other DNA forms such as three-stranded helices and alternative base pairs are also being modelled.

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 development of a version of SAAM30 which executes under the DOS operating system on personal computers which make use of the Intel 80386 and 80486 central processor continues to improve by making use of more modern methods of memory management. Because of the limitations of the newest versions of the DOS operating system we continue to make use of the Phar Lap memory management extensions to compile, link, and execute SAAM30 on this series of computers, however additional memory management techniques have been added to better manage the restricted resources of the 640K limitations of "real memory". SAAM30 runs quickly on these series of computers, but continues to require more than four megabytes of memory and a Weitek, 80387 or Cyrex coprocessor.

This version includes a new and enhanced version of the CONSAM graphics which outputs to the DOS Graphic Software Solution*Computer Graphics Interface Standard (GSS*CGI standard). This choice when combined with the improved memory management of "real mode memory" makes possible the use of separate drivers for the monitor and hardcopy device. This in turn allows the hard copy device to produce camera ready figures well beyond the resolution of the monitor. The PLOT command has been extended so that color, line-type and axis-pair, error bars, closed and open markers, and fill to axis may be specified directly from the command.

The addition of a file browser combined with the inclusion of manual pages documenting 40 of the most used CONSAM commands has been added to the CONSAM software making this documentation available from within CONSAM.

The SAAM project participated in the 1990 NATO conference on advanced pharmacokinetics. The SAAM group organized and participated in workshops on the topics of SAAM/CONSAM in kinetic analysis. A special workshop was again organized to discuss the kinetics of lipid and lipoprotein, the investigation of which centers around the use of SAAM and CONSAM. Several additional SAAMNEWS letters were written in conjunction with the Resource for Kinetic Analysis which sent each newsletter to hundreds of investigators who had indicated that they had an interest in receiving such a news letter.

Because of the recognition of a large number of users and the long history of the use of SAAM and CONSAM, The NCI SAAM project (Zech, Greif) were ask to present a commemorative address honoring Dr. Mones Berman at the Fifth Belgium International Colloquium on Atherosclerosis.

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 hypothesis 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 an responsibilities for more than 29 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 and behavioral aspects of caffeine and other drugs.

A general complex multiple dose model for the pharmacokinetics analysis of drugs was developed in conjunction with the FDA (Zech, Jackson). A new pharmacokinetics descriptor, Cuneq(T), was defined and related to the average non-steady-state drug concentration at time T for a drug with a regular or an irregular dosing interval.

A completely new "X Windows" version of the CONSAM graphics has been written for use with Unix systems with the capability to execute XGKS under X11R4. This capability has allowed the movement of the SAAM and CONSAM software from the VAX to the SUN workstations with the "Open Look" Graphical User Interface.

Image Processing

Analytic methods are continuing to be developed for the GELLAB-II software system with concentration on increasing: resolution, gel image scanning, quantitation accuracy, automatic spot pairing, 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 written software to carry out remote collaborative multimedia image-conferencing that will allow GELLAB-II as well as a broad range of other software to be shared. Objects in an image pointed to by any conferee are noted simultaneously on all other conferees display screens. Remote conferencing could be used in other fields of research where imaging or 2D graphics are used.

Collaborative work has continued with the groups of Dr. P. Sonderegger (U. Zurich), Dr. E. Lester (U. Tenn.), Dr. H. Bauer (Austrian Acad. Sci.), Dr. R. Getzenberg (J. Hopkins U.), Dr. P. Rogan (Hershey Med. Sch.), Dr. R. Levenson (Duke U.). GELLAB-II has been exported to the Univ. of Zurich,

CDC/Atlanta, and the Univ. of Norway. GELLAB-II was ported to the CONVEX super computer and this was used in the analysis of some of the data from the above collaborations. Additional work was required to refine the exported version of GELLAB and to insure smooth updates of exported versions of the software.

Work on nucleic acid structure has continued in a variety of collaborations and directions. The RNA structure analysis system has expanded to include more functionality for analyzing RNA conformations from various perspectives (Shapiro). This has involved the development of new algorithms to explore secondary and tertiary structural motifs. This has been made available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system permits queries of relationships that exist in the RNA secondary and tertiary structural problem domain involving various software/hardware complexes available at FCRC and elsewhere. This includes the control of algorithms that reside on various machines i.e. SUN, SILCON GRAPHICS, VAX, CRAY and CONVEX. A large portion of the system has been ported to a SUN workstation from a SYMBOLICS 3675 to permit broader user access (Shapiro, Kasprzak). The system is currently undergoing testing by selected individuals.

The system has been used to study the fine structural details of the HIV-1 rev responsive element (RRE). The research 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 (Shapiro, Dayton, Konings, Powell, Butini, Maizel). The termination structures of lambda TR2 has also been studied. Results suggest that for the TR2 terminator the stem structures may have multiple roles providing both structure as well as sequence specificity to signal transcription termination (Shapiro, Chang-Cheng, Lynch, Leason, court, Friedman) and is currently being used to study the termination structure of the 5' non-coding regions of poliovirus and its relationship between secondary and tertiary structures (Shapiro, Currey, Maizel). Work has been almost completed on a methodology to predict pseudoknots from RNA secondary structures (Shapiro, Konings).

Exploratory work has continued utilizing an scanning tunneling microscope to visualize the fine structure of molecules, specifically RNA, with some initial encouraging results (Shapiro, Pomerantz, Dayton, Maizel).

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 to test the "binding site barrier" hypothesis are in progress.

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 effect 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) are in progress.

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.

Membrane Structure and Function

The research goals in the Membrane Structure and Function Section (Blumenthal, Puri, Krumbeigel, Clague and Dimitrov) are directed towards an understanding of mechanisms of membrane fusion mediated by viral spike glycoproteins. The mode of action of the envelope protein of HIV of the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus are studied. Specific topics include: 1) development of fluorescent methods to study kinetics and extent of adhesion and fusion using intact and reconstituted virions, and liposomes and cells as targets; 2) development of methods to analyze reconstitution of viral spike glycoproteins; 3) functional reconstitution of viral spike glycoproteins into lipid vesicles; 4) studies of mechanism based on an allosteric model: the role of ligand binding, conformational changes and cooperativity of viral spike glycoproteins in mediating membrane fusion; 5) studies of the effects of modifications of viral spike glycoproteins by pH, temperature, enzymes, and chemicals on their fusogenic activities; 6) studies of the relationship between virus-induced membrane destabilization (permeability changes) and fusion; 7) studies of viral entry into the cell by endocytosis using fluorescent techniques; 8) application of image processing using video-enhanced fluorescent microscopy controlled by a computer to analyze viral entry pathways; 9) examination of the disposition of the fusion protein after the fusion event; 10) identification of possible fusion intermediates; 11) development of methods to study fusion activity of mutants of viral proteins using cloned viral membrane protein sequences expressed in transfected cells; 12) structural studies of viral proteins; and 13) development of methods for using reconstituted viral envelopes as vehicles for specific delivery of materials into cells.

The first step of entry of enveloped animal viruses into cells is by fusion of the membrane of the virus with that of the target cell. This fusion process is catalyzed by viral envelope proteins. We have developed biophysical techniques to study the initial steps of viral envelope protein mediated membrane fusion. We label intact virus or cells with fluorescent dyes and observe the redistribution of those dyes during the fusion process. We study initial steps of HIV envelope protein-mediated membrane fusion, by continuous monitoring of fluorescent dyes during fusion using fluorescence spectroscopy and low light, image enhanced videomicroscopy. In particular we use HIV envelope protein expressed in cells by means of recombinant vaccinia virus and target membranes of defined composition with and without CD4 receptors. In this way we monitor fusion between cells or syncytium formation. The combination of studies employing HIV-expressing effector cells and defined target membranes facilitates the testing of hypotheses regarding the role of different factors in adhesion and fusion. Transmission of retrovirus between cells is thought to be associated with cell membrane fusion. In this way the virus is not exposed to the extracellular space and thereby hidden from the immune response. Thus, membrane fusion is a key element in the pathology of HIV, and an understanding of the mechanism of viral fusion might lead to the development of anti-viral therapeutic agents.

Membrane Biology

Over the past decade we developed a system of methods to examine the nanoanatomy and topochemistry of antigens, receptors and other macromolecules on the outer and the cytoplasmic surface of plasma membranes. This led to the discovery of Fracture-label (1981), Label-fracture (1984), Fracture-permeation (1986), Replica-staining (1988), Fracture-flip (1988), Triton-X Fracture-flip (1989) and Simulcast (1990).

This system of methods is now being used to study problems related to the structure and dynamics of biological membranes. Among our immediate aims are the localization of membrane associated oncogenes and the study of the topology and topochemistry of signal transduction. This will be facilitated by our recent discovery of procedures for visualization of the cytoplasmic surface of the plasma membrane of fibroblasts in culture (Hou) and of immunogold labelling to co-localize mos oncogene and tubulin (Shen). We are also actively pursuing the membrane-pathobiology of bacteremias as induced by lipopolysaccharides released by *E. coli*. Here, our aims are the study of the mechanism of this disease and to devise an early detection test (Risco). The localization of receptors in nerve cells in culture (Caruncho), the transduction of signal during lymphocyte capping (Pavan, Torrisi) and the nanoanatomical and cytochemical differentiation of pathological/non-pathological stages in intracellular parasites (Pimenta, Shen, Dwyer, Sachs) are also being actively pursued. Dr. P. Sirigu, a Visiting Professor from the University of Cagliari, School of Medicine investigates the ultrastructure of the human Meibomian glands. Finally, we continue our investigation of the surface of nanoanatomy and structure of the zonula occludens.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		W01 CB 08300-19 LMMB
PERIOD COVERED	October 1, 1990 to September 30, 1991	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)		
SAAM, Development and Applications for Analogic Systems Realization		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Loren A Zech, M.D.	Senior Investigator Detailed from OD, NHLBI	LMMB, NCI
<u>Other Professional Personnel:</u>		
Peter C. Greif, M.D.	Computer Programmer Analyst	LMMB, NCI
COOPERATING UNITS (if any)		
Dr. Ray Boston, Murdoch Univ., Australia; Dr. Charles Schwartz, Medical College of Virginia, Richmond, VA; Dr. Waldo R. Fisher, Univ. of FL, Gainesville, FL; Drs. H. Bryan Brewer, Daniel Rader, Carlo Gabelli, Alexander Mann, Juergan (cont.)		
LAB/BRANCH		
Laboratory of Mathematical Biology		
SECTION		
Office of the Chief		
INSTITUTE AND LOCATION		
NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.3
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		B
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>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 implement additional memory management techniques. The ability to temporarily clear the "real mode" memory of its contents and then restore it at a later time has increased the capabilities of CONSAM to: 1) make use of additional editors, 2) invoke a "File Browser" to display and search manual pages, 3) include the mouse driver for use in graphics, and 4) retrieve the error information associated with the observed data. This version of SAAM and CONSAM has been compiled and tested on the 80486 style computers running DOS, however it has not yet been tuned to take advantage of the specific capabilities of this hardware.</p> <p>CONSAM graphics has been enhanced to take advantage the mouse driver. This graphic software improved with the addition of background grids, tic markers which face in or out with respect to the axis, and the ability to plot error bars associated with the observations.</p> <p>A completely new "X Windows" version of the CONSAM graphics has been written for use with Unix systems with the capability to execute XGKS under X11R4. This capability has allowed the movement of the SAAM and CONSAM software from the VAX to the SUN workstations with the "Open Look" Graphical User Interface.</p> <p>A unified method of simulating complex dosing regimens using a general dosing program developed using CONSAM provides a rapid method of calculating nonuniform dosing regimens for any linear model of up to 3 compartments. Observed pharmacokinetics data can be curve fitted to obtain individual subject estimates and dosage optimization. Examples were constructed to show the use of the program in the estimation of a new pharmacokinetics parameter, Cuneq(T), which is defined as the average non-steady-state drug concentration at time T for a drug with any regular or irregular dosing interval.</p>		

Cooperating Units (Continued):

Schaefer, Molecular Diseases Branch, NIH/LB; Richard E. Gregg, Squibb Inst. for Med. Res. Princeton, NJ; Dr. Ernest Schaefer, USDA Human Nutrition Center, Tufts Univ. Boston, MA; Dr. Phil Taylor, Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA; Dr. Blossom Patterson, Operations Research Branch, NCI; Drs. Ba-Bie Teng and Allen Sniderman, Royal Victoria Hospital, Montreal, Quebec; Dr. Gilbert Thompson, Hammersmith Hospital, London, England; Dr. Ahmed Kissebah, University of Wisconsin; Dr. Michael H. Green, The Pennsylvania State University, State College, PA; Dr. Gene Barnett, Biometric Research Institute, Arlington, VA.; Dr. Andre J. Jackson, FDA, Rockville, MD.

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 systems uses the MARK and RELEASE utilities (created by TurboPower Software) to free up "real mode memory" after it is finished. Also included with these programs are a variety of useful memory management utilities such as MAPMEM, which details the contents of the lower 640K of memory, and RAMFREE, which indicates how much "real mode memory" is free. While these memory managing utilities can free up memory after the program has run, they cannot do so while the program is running. Thus it has not been possible to run most DOS programs or use your favorite editor from within CONSAM using the SYSTEM or EDIT commands.

A better version of CONSAM designated SWCON accomplishes the MARK and RELEASE functions using the SWAPDOS shareware memory management utility (distributed by Innovative Data Concepts) permitting the user to use their favorite editor and perform other DOS functions requiring large amounts of memory without leaving CONSAM.

The advantage of SWAPDOS is its ability to temporarily clear the "real mode memory" of its current contents and then restore it at a later time. Taking advantage of this capability, SWCON offers you a choice of editors. The user can designate an editor by altering the file SWCON.BAT in the SAAM home directory. All the user need to do is change the line:

```
set saam_edit=edlin
to
set saam_edit=your_editor
```

From that point on, SWCON will invoke your editor when you issue the EDIT command.

Another advantage of SWAPDOS is its ability to temporarily clear the "real mode memory" of its current contents and then restore it at a later time. Taking advantage of this capability, SWCON now offers the ability to invoke a "File Browser" to examine manual pages when using the DICTIONARY command.

Like the editor invoked following the EDIT command, the user can designate a specific browser by altering the file SWCON.BAT in the SAAM home directory. All the user need to do is change the line:

```
set saam_man=listdict
to
set saam_man=your_browser
```

From that point on, SWCON will invoke the users specified browser when they issue the DICT command. In addition, by special arrangement with the author a copy of the file browser LIST is provided. Using this file browser 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.

The PLOT command also has two new switches, C for clear then plot, and R for Retain. For Example:

```
PLOT/R G1_4 Q(11)
```

plots component 11 and returns control to CONSAM while

```
PLOT/C G1_1 Q(2)
```

clears any retained plots then makes a new plot of component 2 before returning control back to CONSAM.

For the user with a mouse we have included possible mouse capability in this version. Looking at the KERNEL.MOW file used with the SWCON version of CONSAM

```
1:DISPLAY
```

can be changed to

```
1:DISPLAY;MOUSE
```

The default is for no mouse.

In addition SUN 4 SPARC version of CONSAM has been developed using the recently released XGKS software available with X windows version 11 release 4. While all that remains to be completed to finish this version is the addition of a file browser and the conversion of the manual pages to the manual format used with Sun OS 4.1

The PLOT command with color, line-type, and axis-pair capabilities has been extended to include the ability to plot error bars. This ability to plot the observed error as error bars on the graph has been added to the command line of

the PLOT command. The new symbol for error bars is ^ followed by U for Up, D for Down, L for Left, and R for right. For example:

```
PLOT G1_1 QC(3^D)
```

plots the observed values for component three with Down error bars, while

```
PLOT G1_1 OO(15^UDLR)
```

plots the observed values on both axes with error bars in every direction.

New additions to the plotting capabilities of CONSAM also include the ability to plot background grids and tic markers both into and away from the axis.

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

We participated in a two week NATO workshop in advanced pharmacokinetics, a major use of SAAM and CONSAM. Plans were made for the Fourth Mathematical Models in Nutrition Conference to take place in Washington, D.C. in conjunction Dr. Meryl Wastney of Georgetown University. In conjunction with the American Institute of Nutrition we presented SAAM and CONSAM at a 1991 FASEB symposium entitled "Computer Modeling in Nutrition" attended by several 100 participants and had an associated computer demonstration area where approximately 500 researchers came to ask questions about SAAM.

Almost 80 copies of the SAAM/CONSAM software have been provided to the scientific community over the past 12 months in an effort to establish other centers in the collaborative effort, as suggested by the scientific counselors. 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 29 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

Major Findings:

(1) In collaboration with Dr. Jackson of the FDA: A simple method of simulating complex dosing regimens increasingly found in the literature using a universal dosing regimen calculated with a general dosing program developed using CONSAM. The program provides a rapid method of calculating nonuniform dosing regimens for any linear model of up to 3 compartments. Observed pharmacokinetics data can also be curve fitted to obtain individual subject estimates, and allow dosage optimization, which is important in a clinical setting. Examples were constructed to show the use of the program in optimizing a theophylline regimen, as well as estimation of a new parameter, $C_{unq}(T)$, which is defined as the average non-steady-state drug concentration at time T for a drug with an irregular dosing interval.

(2) In collaboration with Dr. Schwartz: We have begun to identify and quantify cholesterol pools and transport pathways in blood and liver by making use of the bile fistula perturbation. Using multiple isotopic preparations to accomplish simultaneous labeling of separate cholesterol pools and sampling all components of blood and bile at frequent intervals, we have realized a comprehensive multicompartmental model for cholesterol within the rapidly miscible pool. Data in 6 components (bile acids, esterified cholesterol in whole plasma, and free cholesterol in blood cells, bile alpha and beta lipoproteins) were well predicted using this model. The analysis revealed extensive exchange of free cholesterol between HDL and liver, blood cells and other tissues. There was net free cholesterol transport from HDL to the liver in most subjects. The major organ that removed esterified cholesterol from blood was the liver. A large portion (4211 μmol) of total hepatic cholesterol comprised a pool that turned over rapidly ($t_{1/2}$ of 72 min) by exchanging mainly with plasma HDL and was the major source of bile acids and biliary cholesterol. Only 6% of hepatic newly synthesized cholesterol was used directly for bile acids synthesis. The analysis suggested that newly synthesized cholesterol was partitioned into a large hepatic pool, thought to be the putative plasma membrane free cholesterol, which exchanged rapidly with plasma lipoproteins. Bile acid synthetic rate correlated directly with the size of this large hepatic pool. In conclusion, after hepatic and blood cholesterol pools and transports have been quantitated it is clear that HDL plays a central role in free cholesterol exchange and transport between all tissues and plasma. This is consistent with the reverse cholesterol transport role proposed for HDL. In man, the metabolically active cholesterol pool comprises a large portion of total hepatic cholesterol that, in part, regulates bile acid synthesis.

(3) In collaboration with Dr. Fisher and Dr. Stacpoole: Using in vivo tracer kinetic methodology with a ^3H -leucine tracer, the kinetics of apolipoprotein B (apoB) were measured in 14 studies in 7 heterozygous, familial hypercholesterolemic subjects (F.H.) and in 7 studies in 4 normal subjects. Subjects were studied on three protocols, a weight maintenance diet containing 20% of calories as fat, while receiving 40 mg. per day of lovastatin on the same diet and on a diet consisting of 91% glucose, 8% amino acids and 1% fat. VLDL and LDL were isolated ultracentrifugally and LDL was fractionated into larger and smaller molecular weight subspecies. ApoB was isolated and its specific radioactivity measured, as was the specific activity of plasma

leucine. The data was analyzed and a compartmental modeling was realized using the SAAM computer program.

ApoB synthesis and secretion requires 1.2 hr. ApoB is secreted by three routes: (1) as large VLDL where it is metabolized by a Delipidation Pathway, (2) as smaller VLDL and (3) as small, IDL/LDL particles which probably are predominantly IDL. In F.H. subjects fed a high carbohydrate diet, inducing a relative hypertriglyceridemia, the secretion of large VLDL particles is delayed an additional 1.2 hr, implying an added time required for assembling and secreting large, triglyceride-riched VLDL.

ApoB is metabolized along two pathways. The Delipidation Chain Pathway, as previously described in this laboratory, processed large VLDL to small VLDL, then IDL and subsequently LDL. In addition the IDL pathway channels nascent, small VLDL and nascent IDL particles into LDL. Small VLDL and IDL thus provides a fast pathway for the entrance of apoB tracer into LDL, while the Delipidation Pathway is a slower route for channeling apoB through VLDL into LDL. LDL apoB is derived in approximately equal amounts from both pathways, which predominantly enter into large LDL. Small LDL is primarily a metabolic product of large LDL, and the major loss of LDL-apoB is from the smaller subspecies.

The pathways of apoB metabolism differ in F.H. and normal subjects in two major respects. Normals secrete >90% of apoB as VLDL while one-third of apoB is secreted as IDL/LDL in F.H. Normal controls lose 40-50% of apoB from plasma as small VLDL/IDL while F.H. subjects lose none, metabolizing all of apoB to LDL, thus accounting for the greater transport of LDL-apoB in F.H. compared to normal subjects. In F.H. there is also the known prolongation of LDL residence time.

An endogenous, leucine tracer, biosynthetically incorporated into apoB, permits distinguishing the separate pathways by which the metabolism of plasma apoB is channeled. The resultant metabolic heterogeneity of LDL-apoB provides at least a partial explanation for the molecular weight heterogeneity of LDL seen in F.H.

After realization, the compartmental modeling was used to examine the control aspects of apolipoprotein B (apoB) secretion and catabolism. To accomplish this examination the above experimental results obtained in F.H. and normal subjects on a basal diet and while receiving lovastatin or while being administered a 91% carbohydrate, low fat diet, were compared with previously published kinetic data obtained on seven subjects with hypertriglyceridemia (HTG).

Total apoB secretion in the normal was slightly greater than in F.H. subjects but was increased two-fold above normal in HTG. In the normal and hypertriglyceridemic subjects approximately 90% of apoB is secreted as VLDL which is the primary endogenous, triglyceride transport lipoprotein of plasma. It is reported that in familial hypercholesterolemia hepatic cholesterol content is increased. The finding in these studies that in F.H. about one-third of apoB is secreted as cholesterol ester enriched IDL/LDL while two-thirds is secreted as VLDL is consistent with two concept that the neutral

lipid content of the hepatocyte predicts the core lipid content of secreted lipoproteins.

Since half of apoB was lost from plasma as VLDL remnants in normals and about two-thirds was lost in HTG, LDL-apoB transport was greater in F.H. than in normal and HTG subjects. While in the F.H. subject, LDL-apoB concentration is determined by the superposition increased LDL-apoB transport and reduced fractional catabolic rate (FCR) of LDL-apoB, the latter was the primary determinant. When examining the total plasma apoB balance, neither a high carbohydrate, low fat diet nor lovastatin treatment altered total apoB secretion, carbohydrate feeding shifted the secretion of apoB proportionately away from IDL/LDL sized particles and into large VLDL. These large, presumably triglyceride enriched VLDL required almost one hour of additional synthesis and intracellular processing prior before secretion.

The residence time of LDL-apoB is prolonged in F.H. heterozygote; however, this is reversible as demonstrated by the reduction in LDL residence time to normal following both the carbohydrate and lovastatin perturbations. Both of these perturbations are known to reach a steady state which are known to impair cholesterol synthesis. Based on a reported increased hepatic cholesterol content in the Watanabe rabbit and in humans with familial hypercholesterolemia, it is hypothesized that the F.H. heterozygote uses two mechanisms to reduce hepatic cholesterol: 1) a further down-regulation of hepatic apoB, E receptor expression, below the limits imposed by the receptor deficiency that characterizes this disease; and 2) an increase in LDL-apoB production. Jointly they raise plasma LDL-cholesterol concentration higher than would be expected if the genetic deficiency were the immediate cause of the elevated plasma cholesterol in these patients. Thus by reducing the elevated cholesterol content of the liver towards normal the hypercholesterolemia which characterizes this disease is reduced. The unanswered question in understanding the pathophysiology of F.H. is shifted to, why the liver accumulates cholesterol.

(4) In collaboration with Dr. Blossom Patterson, Operations Research Branch, NCI; Dr. Christane Swanson, Cancer Prevention Studies Branch, NCI; Dr. Orville Lavender, Beltsville Human Nutrition Research Center, USDA: The selenium study was continued to investigate the pharmacokinetics of an organically-bound form of selenium. Using six adults which received a single oral 200 ug dose of ⁷⁴Se as L-selenomethionine (⁷⁴SeMet) tracer in aqueous solution following 3 days of consuming a constant 87 ug of Se/day diet. Subjects remained on the study diet for 12 days of the tracer study while plasma, urine and fecal samples were collected and analyzed using gas chromatography/mass spectrometry. A kinetic model which simultaneously accounted for all of the data included absorption distributed along the gastrointestinal tract, uptake by the liver-pancreas subsystem, enterohepatic recirculation, distribution to two large tissue pools, and transport through 4 plasma components. The tracer was 98% absorbed, and only 15% of the dose was excreted during 12 days. Average turnover time of the plasma components varied from 0.01 to 1.1 days. The turnover time in the liver/pancreas ranged from 1.6 to 3.1 days. Turnover time ranged from 61 to 86 days in the most slowly turning over peripheral tissues. The whole-body residence time was

approximately 5-fold greater than the turnover time of the slowest turning over tissue pool, reflecting substantial reutilization of material. This suggests that conservation of Se ingested as SeMet could be physiologically advantageous if recycled material is directed for incorporated into metabolically active species. In some respects this may represent a fatal cycle in which the physiologic system can produce a constant source of Se-Met to critical tissues in the presence of high variability in levels of intake. Conversely, this recirculation and reutilization may also result in excessive accumulation of Se in tissues.

(5) In collaboration with Dr. Daniel Rader: Apolipoprotein (apo) A-I is the major protein in high density lipoprotein (HDL) and is found in two major subclasses of HDL. Those containing apoA-II (designated LpA-I,A-II) and those not containing apoA-II (designated LpA-I). LipoproteinA-I but not LpA-II,A-I have been proposed as correlates to atherosclerotic risk. The *In Vivo* turnover of the LpA-I and LpA-I,A-II particles have been measured in normal humans. In the first series of studies, radiolabeled apoA-I and apoA-II were reassociated with plasma HDL in injected into normal subjects. Subsequent to the separation of plasma the decay of LpA-I and LpA-I,A-II particles was determined by isolation of these lipoproteins at each time point. In a second series of studies, purified radiolabeled apoA-I was incubated with LpA-I particles and a second radioisotope was used to label the apoA-I reassociated with LpA-I,A-II. Following simultaneous injection the plasma decay resulted in a residence time of 4.4 days for apoA-I injected entering on a LpA-I lipoprotein and a residence time of 5.71 days for apoA-I entering on the LpA-I,A-II lipoprotein. The difference of .8 days in apoA-I residence is very significant and is a major clue for the further development of the compartmental model of apoA-I metabolism.

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

PROJECT NUMBER

201 CB 08303-19 LMMB

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 Membrane Fusion Mediated by Viral Spike Glycoproteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
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SECTION Membrane Structure & Function Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.	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 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.

PROJECT DESCRIPTION

Major Findings:1. *The delay time for influenza hemagglutinin (HA)-induced membrane fusion.*

In kinetic studies on HA-mediated fusion we found that both intact virus and HA-expressing cells display a pH-dependent time lag before the onset of fluorescence dequenching, but of an order of magnitude difference, about -2 seconds versus -20 seconds. We have adopted two strategies to investigate whether the difference in lag time reflects the surface density of acid-activated HA, able to undergo productive conformational change. (i) HA expressed on the cell surface requires proteolytic cleavage with trypsin from an inactive HA0 form. We have limited the extent of proteolysis. (ii) By infection of CV-1 cells with a recombinant SV40 virus bearing the influenza HA. The surface expression of HA is a function of time post infection. For low pH induced fusion of both types of cells with erythrocytes, the delays decrease with increasing HA concentrations with a slope of 0.2 on a log-log plot. The slope of 0.2 means a 3-fold decrease in delay for a 10-fold increase in HA surface concentration. However, the decrease in delay between HA-expressing cells and intact virus is about 10-fold. This means that, normalized to surface concentration of HA, fusion of intact virus is about 3 times more rapid than cell-cell fusion. The physical basis for those differences remains to be elucidated.

2. *The fusogenic activity in cells of influenza HA, and of HA altered in the amino-terminus of HA2 (fusion peptide) by site-directed mutagenesis.*

We examined the mutants M1 and M4, substitutions of GLU for GLY at positions 1 and 4 respectively, and M11, substitution of GLY for GLU at the 11 position (Gething et al, J. Cell Biol. 102, 11-23, 1986). Following expression in Simian cells using SV40-HA recombinant virus vectors, we measured the kinetics of fusion with erythrocytes using a spectrofluorometric fluorescence dequenching assay (Morris et al, J. Biol. Chem. 264, 3972-3978, 1989). In accordance with previous cell biological results M1 did not induce fusion, M11 showed the same pattern as the wild type (WT), and M4 showed a shift of about 0.4 in pH threshold. The overall kinetics of M4 and WT were similar in lag times for the onset of fusion, rise times and extent. A closer examination of the kinetic processes, however, revealed that the lag time could be dissected into at least two components: a commitment time of 5-10s, and a maturation time of 20-30s. Although the maturation times for WT and M4 were similar, the commitment time was more rapid with M4. These results are consistent with the notion that only the relatively rapid components in the overall fusion reaction depend on the structure of the fusion peptide, whereas the slower processes are governed by other factors.

3. *A dissection of steps in influenza HA-mediated fusion.*

Incubation of influenza virus-erythrocyte complexes at pH 4.8, 4°C resulted in the establishment of a fusion committed state with the following characteristics: a) Subsequent incubation at pH 7.4, 37°C led to fusion; b)

the kinetics and extent of this neutral fusion were dependent on the time period of the low pH incubation; c) the committed state was effectively retained at pH 7.4, 4°C for long time periods (> 1 hour); d) the committed state was insensitive to 20mM DTT, neuraminidase or trypsin (which react with HA1), but incapacitated by the proteolytic enzymes, proteinase K and thermolysin, which removes the 1-27 residue section of HA2. We conclude that the fusion committed state no longer requires the HA1-sialic acid interaction for binding, nor the presence of HA1 for the fusion reaction to proceed, but that HA2 is a necessary component in the later stages of the fusion pathway.

4. *Redistribution of viral lipid and protein upon influenza virus fusion.*

We examined by video microscopy the redistribution of R18 and FITC-labeled HA upon incubation of influenza-erythrocyte complexes at low pH and different temperatures. At temperatures > 15°C and pH 4.9, lipid and protein 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 remained with the virus. These studies are consistent with the formation of fusion junctions which allow passage of lipid while the protein is still retained with the virus.

5. *Role of steric and electrostatic barriers in fusion of vesicular stomatitis virus with cells.*

Desialylation of VSV significantly enhanced the extent of fusion with Vero cells but affected neither the pH-dependence nor the binding of VSV to Vero cells. The enhanced fusion of asialo-VSV was observed both at the plasma membrane as well as via the endocytic pathway. Asialo-VSV showed enhanced fusion activity with negatively charged (phosphatidylcholine (PC)-phosphatidylserine (PS), 1:1 mole ratio) as well as uncharged (PC) liposomes indicating that intermembrane electrostatic effects did not play a significant role in viral fusion. To examine factors which contribute to the extent of fusion we analyzed the various activation and desensitization reactions that take place as a result of low pH triggering of VSV prebound to the target membrane. Lag times for the onset of fusion were similar for VSV and asialo-VSV, indicating that desialylation did not affect the activation reactions. However, exposure of VSV bound to target membranes at pH 6.5 for 400 sec led to considerable desensitization, whereas little desensitization was seen after desialylation of VSV. These results are consistent with the notion that productive fusion of VSV with cells occurs when pH-dependent conformational changes of the envelope protein are induced while the virus is in the appropriate position with respect to the target membrane.

6. *Fusion of vaccinia virus with cell membranes.*

The membrane fusion activities of the isolated single-enveloped intracellular form of vaccinia virus (INV) and the double-enveloped extracellular (EEV) were studied using the R18 dequenching assay. Both form fused at neutral pH with Hela cells, suggesting that fusion occurs at the plasma membrane during virus entry. A monoclonal antibody against the 14 kDa envelope protein of INV completely suppressed the initial rate of fusion of INV but had no effect on

the fusion activity of EEV, suggesting that vaccinia virus encodes two or more fusion proteins. Cells infected with vaccinia formed syncytia when briefly incubated at pH 6.4 or below, indicating that an acid-activated viral fusion protein is expressed on the cell surface. However, the intact virus did not display increased fusion activity at acid pH, suggesting that the acid-dependent fusion factor is not incorporated into virions or that its activity is masked.

7. *A phenomenological model for viral fusion.*

We developed a model, based on physical kinetics principles, which allows description of one of the components of the delay in fusion kinetics as function of the physical properties of the interacting membranes and the medium between them. The basic assumptions are: i) the fusion trigger decreases the energy barrier preventing fusion and ii) membranes are in appropriate structural position to move and make molecular contact. Based on these assumptions we derived a formula describing the lag times in fusion, which is in reasonable agreement with our experimental data for influenza, VSV and Sendai virus. One of the estimates, based on this model, showed that the free energy change, caused by the exposure of the hydrophobic peptides, is sufficient to overcome the hydration barrier to fusion and cause the membranes to move against each other and make local molecular contact. The main energy barrier then seems to be related to structural reorganizations of the lipid bilayers of the approaching membranes. This is in agreement with our observations that the activation energies for fusion of HA and VSV are of the same order of magnitude, which is about the same as the activation energy for lateral mobility of lipids. It is also of the same order of magnitude as for other fusion systems, e.g. fusion induced by electric fields. This implies that during the initial steps of events leading to fusion, the exposure of the fusion peptides can lead to efficient membrane approach and merging without the necessity for insertion of the fusion peptides either in the target or in the viral membrane.

Description of AIDS Research

8. *Initial stages of HIVenv-mediated cell fusion monitored by a new assay based on redistribution of fluorescent dyes.*

To gain insight into mechanisms of HIVenv-mediated membrane fusion, we developed a new assay for studying the initial events. The assay is based on the redistribution of fluorescent markers between membranes and cytoplasm of adjacent cells examined by means of fluorescence video microscopy. Membrane fusion between HIV-1 envelope glycoprotein (gp120/41) expressing effector cells and CD4⁺ target cells was observed 90 min after the association of cells, whereas the first syncytia only became apparent after 5h. Moreover, membrane fusion events were observed under conditions where no syncytia were detected, for example, when the effector:target cell ratio was greater than 100:1, or less than 1:100. A significant number of cells with fused membranes were not involved in the syncytia. In order to determine whether quantitative differences in receptor expression might influence the extent of membrane

fusion, we used laboratory selected variants of CEM cells that differ in their expression of CD4. We found that CD4 is required on the target membrane for HIVenv-mediated membrane fusion, but its extent is only partially dependent on CD4 surface concentration. The ability of those CEM variants to take part in HIVenv-mediated membrane fusion did not correlate with their capacity to form syncytia. These findings indicate that additional steps are needed to form syncytia after membrane fusion.

9. Fusion of intact HIV-1 with cells.

We developed a protocol for measuring fusion of intact HIV with cells, based on the fluorescence dequenching assay. The new refinements are: i) labeling of the virus with small amounts of fluorescence dye, ii) separation of the labeled virions from the dye by two sets of filters, iii) prebinding of the viruses to cells in small volumes and iv) simultaneous measurements of controls in a rotating four-cuvette system. This new protocol reflects specific features of HIV and its fusion with cells: fragility of the virus, slow and inefficient fusion, possibilities for artifacts. One major advantage over a previously published protocol is that it allows continuous monitoring of the fusion reaction. The protocol also enabled us to measure the extent of HIV binding to cells. The major new finding is that HIV fuses with cells after a lag time (delay), which depends on the cell type (120, 150 and 100s for MT4, H9 and 12D7 respectively). The fusion yield reached half of its maximal value about 10 min later, and was completed within 30 min. The highest fusion yield was measured for MT4 cells, followed by H9 and 12D7 cells, which correlates with their susceptibility to infection. We also found that for MT4 cells: 1) fusion did not occur at 4°C 2) Fusion takes place at 18°C. The lag times at that temperature are longer and the fusion yield smaller than at 37°C. 3) The monoclonal antibody OKT4A blocked fusion partially. It also decreased the extent of binding of the virions to cells. 4) A monoclonal antibody which blocked fusion partially did not significantly affect the virus binding. 5) Preincubation of the virus with recombinant GP120 for 10 h did not increase the fusion yield. 6) Glutaraldehyde induced extensive fusion at room temperature. This raises the possibility for artifacts in electron microscopy observations of viral fusion. 7) The number of virions bound per cell is of the order of hundreds. It is not efficient and may be to a large extent non-specific.

10. Binding and fusion of plasma membrane CD4 vesicles with cells expressing gp41/gp120.

We prepared vesicles from Molt-3 cells (a T4 lymphocyte cell line) and from HeLa cells infected with recombinant Vaccinia containing the gene for CD4 (yielding approx. 10 fold higher expression of CD4 receptor on the cell surface) by hypotonic lysis of the cells. The CD4 vesicles were characterized by (a) encapsulation of a water soluble probe, BCECF, (b) surface antigen staining by monoclonal antibody FITC-OKT4 and (c) specific binding to GP120-expressing cells. Using low light level video microscopy we detected fluorescence on vaccinia-CD4-infected HeLa vesicles but not on Molt3 vesicles after the staining with FITC-OKT4. However, after labeling the vesicles with the fluorescent dye, R18, we observed specific binding of both types of

vesicles to HIVenv-expressing cells. Redistribution of R18 from the CD4 vesicles to the HIVenv-expressing cells was observed by low light level video microscopy upon incubation at 37°C consistent with fusion of membranes.

11. *Neutralization of HIV by sCD4 is determined by the kinetics of competition for HIV between cell surface CD4 and sCD4.*

sCD4 neutralizes HIV-1 infection more effectively when preincubated with the virus prior to infection of CD4⁺ cells. While 1 µg/ml sCD4 was sufficient for neutralization after 30 min preincubation with the virus, 10 µg/ml sCD4 was required for neutralization without preincubation. Kinetic studies of preincubation at different sCD4 concentrations indicate that the interaction of sCD4 with gp120 on the virus leads to a slow inactivation process. If HIV-1, sCD4 and CD4⁺ cells are incubated simultaneously, the cell surface CD4 has a kinetic advantage over sCD4 for the viral envelope glycoprotein. These findings may help in designing new approaches to treat AIDS patients with sCD4.

12. *Inhibition of HIV_{env}-mediated syncytia formation of CD4⁺ cells by sCD4 requires continued presence of sCD4.*

Although syncytia formation between HIVenv-expressing cells (using recombinant vaccinia) and CD4⁺ cells was completely inhibited in the continued presence of 1 µg/ml sCD4, no inhibition was observed if the HIVenv-expressing cells were preincubated with sCD4, followed by washing and mixing with CD4⁺ cells. To examine whether this was due to desorption of sCD4 upon washing, we measured the kinetics of adsorption/desorption of sCD4 to the cell-associated gp120 by flow cytometry analysis using FITC-conjugated OKT4. At 37°C the binding kinetics of sCD4 (2 µg/ml) to HIVenv-expressing cells was biphasic in that the binding reached a maximal value after about 30 min of incubation followed by slow decrease. We interpret the decreasing phase as sCD4-induced shedding of gp120 from gp41 on the cell surface in accordance with previously published data (Moore et al, Science 250: 1139). At 4°C the binding kinetics was 10-fold slower, and no decrease was seen. These results indicate that shedding of gp120 is not sufficient for inhibition of the HIVenv fusion activity, and that viral neutralization requires the continued presence of sCD4.

13. *HIV-1 infection kinetics in human T-cell lines.*

HIV-1 infection of continuous lines of T cells in tissue cultures at low multiplicity of infection (MOI) commonly proceeds in three stages: i) low viral production, ii) sharp increase in viral concentration to reach the peak of infection and iii) lack of viral production. The period of time between the onset of infection and the infection peak (lag time or delay in infection of cell suspensions) increases with decreasing MOI. We found that for 12D7 cells (a subclone of A3.01 cells) every log in MOI decrease corresponds to about 2 days increase in the delay of infection peak. This observation and the overall kinetics of increase of virus concentration were described by a phenomenological model, based on the assumptions that the virus production is

proportional to the number of infected cells and that the rate of infection is proportional to the number of infectious virions and uninfected cells. The model permits the quantitation of viral infectivity by measuring the periods of time needed to reach the peak of viral infection and the characteristic times of sharp increase in viral concentration. It may have implications for the progression of HIV-1 infection and transmission in vivo, including effects of antiviral agents.

Publications:

Puri A, Booy FP, Doms RW, White JM, Blumenthal R. Conformational changes and fusion activity of influenza hemagglutinin of the H2 and H3 subtypes: effects of acid pretreatment. *J Virol* 1990;64:3824-3832.

Walter A, Eidelman O, Ollivon M, Blumenthal R. Functional reconstitution of viral envelopes. In: Wilschut J, Hoekstra D. eds. Cellular membrane fusion: Fundamental Mechanisms and applications of membrane fusion techniques. New York: Marcel Dekker, Inc, 1990:395-418.

Doms RW, Blumenthal R, and Moss B. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane: *J Virol* 1990;64:4884-4892.

Blumenthal R, Loyter A. Reconstituted viral envelopes: "Trojan Horses" for delivery of macromolecules into cells. *Trends in Biotechnology* 1991;9:41-45.

Clague MJ, Schoch C, Blumenthal R. Delay time for Influenza Virus Hemagglutinin membrane fusion depends on hemagglutinin surface density. *J Virol* 1991;65:2402-2407.

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, in press.

Clague MJ, Knutson J, Blumenthal R, Herrmann A. The interaction of influenza hemagglutinin amino terminal peptide with phospholipid vesicles: A fluorescent study. *Biochemistry* 1991, in press.

Blumenthal R, Schoch C, Puri A, Clague MJ. A dissection of steps leading to viral envelope protein-mediated membrane fusion. *Ann NY Acad Sci*, in press.

Puri A, Clague MJ, Schoch C, Blumenthal R. Kinetics of fusion of envelope viruses with cells. *Methods Enzymol*, in press.

Morris SJ, Zimmerberg J, Sarkar DP, Blumenthal R. Kinetics of cell fusion mediated by viral envelope proteins. *Methods Enzymol*, in press.

Dimitrov DS, Blumenthal R. Kinetics of intermembrane interactions leading to fusion. In: Ohki S ed., Cell and model membrane interactions, New York: Plenum Press 1991, in press.

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

PROJECT NUMBER

Z01 CB 08320-16 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Peptide Conformations

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

Robert Jernigan, Ph.D. Deputy Chief, LMMB, NCI

Other Professional Personnel:

Shou-ping Jiang	Visiting Scientist	LMMB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI

COOPERATING UNITS (if any)

Dr. J. Ferretti, Laboratory of Chemistry, NHLBI.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

0.8

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

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

B

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

The solution conformations of two tachykinins, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂), physalaemin (pGln-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH₂), senktide (Asp-Phe-Phe-Gly-Leu-MetNH₂) and septide (Asp-Phe-Phe-Pro-Leu-Met-NH₂) have been studied here with a combination of NMR 2-dimensional NOE's and semi-empirical energy calculations. The number of possible conformations of these small peptides is extremely large, and requires a large computational effort to locate the low energy conformations of these peptides. Energy calculations are carried out for fragments to find low energy conformations by energy refinement. Then, these are used as possible starting conformations for the whole molecule. Thus, the starting conformations of the whole molecule are chosen by combining the low energy conformations of each fragment. Following this, the total conformational energies are calculated and energy refinements carried out. Conformations are then selected that are consistent with the NMR 2-dimensional NOE data. The receptor selectivity of these peptides appears to be related to their conformation. Results suggest these bindings might be enhanced by rigidifying the peptides in an appropriate way.

PROJECT DESCRIPTION

Major Findings:

In water, substance P and physalaemin prefer to be in an extended conformation. However, in methanol, their conformations are a mixture of β -turn conformations in dynamic equilibrium. Solvent titration data and chemical shift temperature coefficients complement the NMR estimates of interproton distances by assisting in the location of hydrogen bonds and identification of dominant conformational states. The C-terminal tetrapeptide appears to have the same conformation for both substance P and physalaemin. In physalaemin, the middle of the peptide is constrained by the formation of a salt bridge.

In methanol, the lowest energy conformation of senktide is found to be an α helical structure with additional hydrogen bonds between the carbonyl oxygen of Phe2 and the amide groups of Gly and Leu, and other possible hydrogen bonds, such as between CO(Phe3)-NH(NH2) and CO(Asp)-NH(Leu). The NH group of Phe3 points toward the outside in its low energy conformation. The planes of the aromatic rings of Phe2 and Phe3 point in different directions and are almost perpendicular to each other. All tachykinins have the Gly-Leu-Met-NH2 fragment in common and Substance P has Phe-Phe-Gly-Leu-Met-NH2 fragment in the C terminal sequence. The α helical conformation is one of the lowest energy conformations for senktide. Substance P may be in α helical conformation at the Phe-Phe-Gly-Leu-Met-NH2 on its C terminal end, and at least partially α helical conformations are common to all tachykinins.

The lowest energy conformation for septide is very different from senktide because of the replacement of glycine in senktide with proline in septide. None of the low energy conformations have an α conformation just before the proline. But β conformation and some others followed by proline exist among low energy conformations.

Publication:

Sumner SCJ, Gallagher KS, Davis DG, Covell DG, Jernigan RL, Ferretti JA. Conformational analysis of the tachykinins in solution: Substance P and physalaemin. *J Biomol Str Dyn* 1990; 8:687-707.

Bohacek RS, Strauss UP, Jernigan RL. Configurational statistics of methyl vinyl ether-maleic anhydride copolymer: Selection of important atomic interactions and conformations. *Macromolecules* 1991; 24:731-739.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 CB 08341-13 LMMB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
John N. Weinstein, M.D., Ph.D.	Chief, Theoretical Immunology Section	LMMB, NCI,
<u>Other Professional Personnel:</u>		
H. Robert Guy, Ph.D.	Senior Staff Fellow	LMMB, NCI
Kai-Li Ting, Ph.D.	Computer Programmer	LMMB, NCI
Vellarkad Viswanadhan, Ph.D.	Visiting Scientist	LMMB, NCI
Benjamiin Denckla	Summer Student	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 MAN-YEARS: 1.5	PROFESSIONAL: 1.2	OTHER: 0.3
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		B
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
To understand the molecules important to our projects on the pharmacology of biological ligands (Z01 CB 08366-07) and combination therapy (Z01 CB 08392-03), we have explored several innovative ways of analyzing protein structure-function relationships:		
1. <u>HAL, HALP, & HALCO.</u> 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. <u>A joint prediction algorithm.</u> 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. <u>3-dimensional quantitative structure-activity relationship (3D-QSAR) studies.</u> 3D-QSAR analysis was done using a set of 20 nucleoside transport inhibitors. The nucleoside transport protein is a major target for dipyrindamole, which we have found to potentiate the activity of AZT against HIV-1 (see project #201 CB 08392-03). Clinical trials of AZT/dipyrindamole 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. <u>Thermodynamic cycle perturbation (TCP) on HIV-1 protease-inhibitor complexes.</u> 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 08392-03).		

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 08392-03)).

(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 08392-03) 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 08392-03). 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, Weinstein JN, Elwood PC. Secondary structure of the human membrane-associated folate binding protein using a joint prediction approach. *J Biol Structure and Dynamics* 1990; 7:985-1001.

Viswanadhan VN, Ghose AK, Szebeni J, Weinstein JN. Mapping the binding site of the nucleoside transporter protein: A 3D-QSAR study. *Biochim Biophys Acta* 1990; 1039:356-366.

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

PROJECT NUMBER

Z01 CB 08363-09 LMMB

PERIOD COVERED October 1, 1990 to September 30, 1991

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

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

H. Robert Guy, Ph.D. Senior Staff Fellow LMMB, NCI

Other Professional Personnel:

G. Raghunathan, Ph.D. Visiting Fellow LMMB, NCI

S. Durrell, 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 MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0
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CHECK APPROPRIATE BOX(ES)

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

B

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

The 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: δ lysin, magainins, cecropins, alamethicin, pardaxin, annexins, and voltage-gated potassium channels.

The simplest structures we have been analyzing are a series of peptides (δ lysin, magainin, PGLa, alamethicin, and pardaxin) that range in size from 21 to 33 residues. The secondary structures of all these peptides appear to be primarily helical; however, the C-terminus portion of pardaxin may be a β strand that, when assembled with other pardaxin peptides, forms the narrow portion of the channel. Because of their relatively small size, more extensive calculations can be performed on these peptides than on the larger proteins described below.

We have continued to develop structural models of larger integral membrane proteins. The protein families for which we have developed phase three models are voltage-activated potassium channels and annexins. These models predict the residues responsible for causing the channels to open and close and those that determine the ion selectivity properties of the pore.

PROJECT DESCRIPTION

Major Findings:

Most of our time this year was spent using computer graphics and molecular mechanics calculations to develop more precise models of the proteins on which we have been working.

A protein structural motif in which an eight stranded parallel β barrel is surrounded by eight α helices has been reported for over twenty different soluble proteins. This structural motif may be common also for ion channel forming proteins. We have developed models of three families of channels using this or a similar motif. The simplest of these is pardaxin, which is a 33 residues peptide. In our model pardaxin can be divided into three segments: the N-terminus (residues 1-11) which is predominately helical and has two positive charges, the middle segment (residues 13-26) which forms an amphipathic α helix with one positive charge, and the C-terminus (residue 28-33) which forms a β strand with two negative charges at its end. Eight monomers are postulated to form a channel in which the N-termini form a circular ring on the outer membrane surface, the middle helices span the inner portion of the membrane, and the C-termini β strands form a β barrel in the center of the assemble (their negative charges form salt bridges with the positive charges on the N-termini and middle segments.) The energies of this model has been calculated and compare favorably with energies calculated for alternative models of pardaxin. We have included water inside the pore in these calculations and have performed molecular dynamic simulations of the protein-water complex.

We have developed a preliminary model for the calcium channel forming protein synexin using a similar structural motif. Synexin, and homologous proteins, have over 300 residues but can be divided into four homologous domains. The secondary structure we predicted for our model of the membrane channel is similar to that determined by X-Ray crystallography for the water soluble form of the homologous endonexin protein; all twenty α helical regions we predicted occur in the crystal structure. We have developed a model for the conformational changes that occur when the protein moves from water into the membrane. We are collaborating with Harvey Pollard's group to test this model.

We have continued our efforts to models the structure of voltage-gated sodium, calcium, and potassium channels. Our controversial prediction that the ion selective pore of the channel would be formed by sequentially short segments that may span only part of the membrane, possibly as β strands, has been confirmed by mutagenesis experiments in three laboratories. In our current models an eight stranded antiparallel β barrel is surrounded by eight α helices which are surrounded by sixteen α helices. The activation gating mechanism involves movement of the positively charged S4 helix.

Little is known about how proteins insert into membranes. On the basis of our modeling we have proposed new mechanisms for how peptides that form amphipathic α helices may interact with membranes and form channels. In our models of δ lysin, the helices assemble antiparallel to each other on one

surface of a membrane to form rectangular 'raft-like' structures that are hydrophobic on one surface and hydrophilic on the opposite surface. In our model of pardaxin assembly, the 'rafts' have radial symmetry and insertion occurs when residues in the center of the 'raft' cross the remaining portion of the membrane. In our models of magainin and cecropins, monomers form antiparallel dimers that in turn assemble to form a hexagonal lattice on the surface of the membrane. Unlike the 'rafts' the peptides do not displace all the lipids of the outer monolayer; lipids fit between the peptides in the regions of three-fold symmetry. The pores form in the regions of six-fold symmetry.

We have also begun a collaboration with Robert Blumenthal to develop structural models of how the influenza virus membrane fuses with target cell membranes. To model this process, we modeled the transmembrane portion of the influenza hemagglutinin (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.

Publications:

Raghunathan G, Seetharamulu P, Brooks B, Guy HR. Models of δ hemolysin membrane channels and crystal structures. *Proteins: Structure, Function, and Genetics* 1990;8:213-225.

Guy HR, Conti F. Pursuing the structure and function of voltage-gated channels. *Trends in Neuroscience* 1990;13:201-206.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 CB 08366-08 LMMB
PERIOD COVERED		
October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
John N. Weinstein, M.D., Ph.D. Chief, Theoretical Immunology Sect. LMMB, NCI		
<u>Other Professional Personnel:</u>		
Kenji Fujimori, M.D.	Visiting Fellow	LMMB, NCI
William van Osdol, Ph.D.	Staff Fellow	LMMB, NCI
Jun Sato, M.S.	Guest Researcher	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 MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		B
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)		
<p>Before a monoclonal antibody (or other biological ligand) can label or kill a tumor cell, it must first reach that cell. For portions of a tumor 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 thus far include the following: (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 may sometimes be preferable. (2) The flux of non-binding control Ig is much less likely to be limited by diffusion or convection. Nonspecific Ig's penetrate more deeply and more quickly into the tumor. (3) Even with saturable binding (but not metabolism), the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. (4) Metabolism will decrease the relative "C times T" exposure of cells farther from the blood vessel. This may be a major barrier to effective treatment of solid tumors with ligand molecules.</p> <p>Predictions of the model are being tested using a subcutaneous tumor in guinea pigs and micrometastases of human tumors in nude mice. The distribution of antibody is being determined by fluorescence techniques and autoradiography. Concepts arising from this study have been applied to the design of clinical studies with monoclonal antibodies.</p> <p>In addition to immunoglobulin and other ligands (e.g., soluble CD4) as <u>administered agents</u>, we are considering the physiology of <u>endogenous</u> molecular species including the lymphokines and growth factors.</p>		

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 be compared; (8) Bivalent binding can be simulated; (9) the global pharmacology can be integrated with the percolation calculations; (10) the computer program package developed ("PERC") is broadly capable of handling various ligands of differing valence. (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 immunconjugates.

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:

Weinstein JN. Antibody lymphoscintigraphy. In: Goldenberg DM, ed. Cancer imaging with radiolabeled antibodies. Boston, MA: Kluwer Academic, 1990;365-385.

Weinstein JN, Fujimori K. Antibody-mediated drug delivery. In: Hooke JB, Poste G, eds. Protein design and the development of new therapeutics and vaccines. New York: Plenum Press, 1990;359-370.

Weinstein JN, Fujimori K. Predictive pharmacology of monoclonal antibodies. In: Bannasch P. ed. Cancer Therapy: New Trends. Berlin: Springer-Verlag, 1989;197-199.

Fujimori K, Covell DG, Fletcher JE, Weinstein JN. A modeling analysis of monoclonal antibody percolation through tumors: A binding site barrier. J Nucl Med 1990;31:1191-1198.

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 Research, in press.

Weinstein, JN, Fujimori, K. The pharmacokinetics of monoclonal antibodies and derived peptides. In: Sundwall A. ed. Preclinical evaluation of peptides and recombinant proteins. Stockholm: Skogs Grafiska AB, 1990;35-41.

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 occasionally utilize detailed atom-atom calculations, we feel the development of higher order principles of molecular structure is essential if we are to achieve a complete understanding of all of the complexities of biological macromolecules themselves, as well as their interactions with other small molecules, other macromolecules and their assembly into biological structures. This project is aimed in that direction.

We have collected statistics on globular proteins from their X-ray structures, counting the amino acid residues that are frequently found near one another in the three dimensional structures. These were obtained in the following way: a lattice model is used in which each residue type has a coordination number. If a specific residue has an incompletely filled coordination shell, then it is assumed to be filled with equivalent water molecules. These derived contact energies follow intuition with the most favorably interacting pairs being hydrophobic residues. This type of energy has a direct bearing on folding and substantially reflects the effects of water on folding. There is a segregation of hydrophobic and hydrophilic residues. These values reflect the actual situation inside proteins and provide a tool that can be applied to a variety of problems and, in the problem at hand, can be used to assess the relative overall quality of different conformations.

The present 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 to use simulated annealing and Monte Carlo to achieve conformational transitions 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 calculations.

Also these methods are being applied to the protein design problem to consider development of an inhibitor to the HIV-1 Protease.

Publications:

Jernigan RL, Covell DG. Coarse graining conformations: a peptide binding example. In: Beveridge DL, Lavery R, eds. Theoretical biochemistry and molecular biophysics. Vol. 2: Proteins. Guilderland, New York: Adenine Press, 1991:69-76.

Jernigan RL, Covell DG. Compact protein conformations. In: Renugopalakrishnan V, Carey PR, Smith ICP, Huang S-G, Storer AC, eds. Proceedings, Expanding frontiers in polypeptide and protein structural research. 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 08371-08 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Conformational Variation of DNA and DNA-Protein Binding

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

Robert Jernigan, Ph.D. Deputy Chief, LMMB, NCI

Other Professional Personnel:

Gopalan Raghunathan, Ph.D.	Visiting 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

COOPERATING UNITS (if any) Dr. Ruth Nussinov, Inst. of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; Dr. Jacob Mazur, Gary W. Smythers, and Dr. David Wang, PRI, Frederick, MD; NIMH; Akinori Sarai, RIKEN Institute; Daniel Camerini-Otero, NIDDK; H. Todd Miles, NIDDK.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.3

PROFESSIONAL:

3.3

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

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

B

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

Conformational analysis of DNA shows that the origin of the B-form double helix can be substantially attributed to the atomic charge pattern in the base pairs. The pattern favors specific helical stacking of the various base pairs. The base pairs alone, without the sugar-phosphate backbone have a strong tendency to be helical. The backbone appears to play a relatively passive role in determining the helix form of DNA. Both electrostatic and van der Waals interactions play a role, but electrostatic interactions are particularly important in A-form DNA.

The asymmetries of conformational fluctuations were calculated. These directional preferences were used to a construct specific sequence models for bending DNA around a protein of known structure, based on DNA footprinting. The TATA box is especially flexible because of alternating large fluctuations in roll and twist; this suggests the role of TATA as a "swivel" joint.

Models of a specific sequence DNA bending around a protein were built, based on footprinting data and flexibility calculations. Other models have been built for tight hairpin loops and for various triple helices.

PROJECT DESCRIPTION

Major Findings:

We have studied the sequence dependence of DNA conformational transitions between B- and A-forms. The role of intramolecular interactions between base pairs, without backbone, has been examined for the conformational transitions between B- and A-form helices. The base pairs themselves usually have intrinsic conformational preferences for B- or A-forms. Calculation of all ten possible base steps shows that the base combinations, CC (or GG), GC, AT, and TA, have tendencies to assume A-conformations. Results show that it is particularly easy to slide along the long axis of the base pair for these steps, with AT and CC showing especially flat energies. However, a preference for B- or A-conformation depends on the electrostatic energy parameters, in particular, on dielectric and shielding constants; A-conformation is preferred for low dielectric constant or low shielding. Both A- and B-conformations are stabilized mainly by electrostatic interactions between favorable juxtaposed atomic charges on base pairs. However B-conformation generally has more favorable van der Waals interactions than A-form. These sequence-dependent conformational preferences and environmental effects show substantial agreement with experimental observations.

Also, we have studied the sequence dependence of flexibility and its anisotropy along various conformational variables of DNA base pairs. Our results show the AT base step to be very flexible along twist coordinate. On the other hand, homonucleotide steps, GG(CC) and AA(TT), are among the most rigid base steps. For a roll motion that would correspond to a bend, the TA step is most flexible, while the GG(CC) step is least flexible. The flexibility of roll is quite anisotropic; the ratio of fluctuations toward the major and minor grooves is the largest for the GC step and the smallest for the AA(TT) and CG steps. Propeller twisting of base pairs is quite flexible, especially of A•T base pairs; propeller twist can reach 19° by thermal fluctuations.

The methods can be utilized in modelling specific sequence DNA bending. Other aspects being investigated include alternative base pairing - Hoogsteen and reverse Hoogsteen, and their effect on a given helical structure.

Publication:

Goodman S, Yang C-C, Nash H, Sarai A, Jernigan RL. Bending of DNA by IHF protein. In: Sarma RH, Sarma MH. eds. Structure and methods: DNA protein complexes and proteins. Vol. 2, NY: Adenine Press, 1990;51-62.

Ragunathan G, Jernigan RL, Ting K-L, Sarai A. Solvation effects on the sequence variability of DNA double helical conformations. J Biomol Str Dyn 1990; 8:187-198.

Raghunathan G, Jernigan RL, Miles HT, Sasisekharan V. Conformational feasibility of a hairpin with two purines in the loop. 5'-d(GGTACIAGTACC-3'. Biochemistry 1991; 30:782-788.

Zhurkin VB, Ulyanov NB, Gorin AA, Jernigan RL. Static and statistical bending of DNA evaluated by Monte Carlo simulations. Proc Natl Acad Sci USA, in press.

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

PROJECT NUMBER
 Z01 CB 08380-07 LMMB

PERIOD COVERED
 October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 Molecular Structure of Animal Viruses and Cells by Computational Analysis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
 Jacob V. Maizel, Jr., Ph.D. Chief, Laboratory of Mathematical Biology, LMMB, NCI

Other Professional Personnel:

John Owens	Computer Specialist	LMMB, NCI
Lewis Lipkin, M.D.	Medical Officer	LMMB, NCI
Ann Barber, M.D.	Senior Staff Fellow	LMMB, NCI
Bruce Shapiro, Ph.D.	Computer Specialist	LMMB, NCI
Danielle Konings, Ph.D.	Visiting Fellow	LMMB, NCI

COOPERATING UNITS (if any)
 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 MAN-YEARS: 4.2	PROFESSIONAL: 3.2	OTHER: 1
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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.)

Complex macromolecular processes and the 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 needed by these systems to function.

Gene and protein sequences of HIV, and related viruses are analyzed by computer methods to find relationships among them and to other known and hypothetical proteins. Predicted secondary structures of their RNAs vary in correlation with pathological and sequence differences.

Foot-and-mouth viruses and adenoviruses are studied with a goal to understanding early events in virus replication wherein the cell's metabolism is subverted to viral functions, and late events during which assembly and morphogenesis occurs. Early viral proteins, whose existence was known from biochemical studies, have been analyzed by comparing their sequences to cellular proteins of known function.

Computer analysis of proteins and nucleic acids are developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, human immunodeficiency viruses. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Computer programs are developed locally and elsewhere for application on Cray XMP, VAX and graphic workstations to perform sequence analysis and structure predictions. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Roles for genes and proteins are deduced by comparison with databases of sequences of known function and structure.

PROJECT DESCRIPTION

Major Findings:

Multiple sequence alignment for the detection of signals: Multiple sequences for related genes provide a powerful data set for detection of structural entities that are involved in recognition by enzymes or regulatory elements, and for understanding common structural motifs. A dynamic program, ANA, for creating alignments of hundreds of sequences, extracting the features and assessing the statistical significance of such alignments has been developed on a Silicon graphics workstation using the C language. Extensions to this program permit rapid assessment of the probability of chance occurrence for a given feature using a method based on binomial probability theory. A similar but non-dynamic program has been developed from the existing FEATUR program in pascal on the VAX computer. Using these programs a number of observations of consensus signals in the vicinity of splice sites, promoter sites and other items contained in the GENBANK "feature" table have been compiled. Analogous programs have been developed for use with amino acid sequences. They are used to find common structural domains between HIV proteins and known families of enzymes such as G-proteins and reverse transcriptases and structural proteins, other viral sequences and potential antigenic sites. A new program, "MOTIFN", finds patterns with spaced features common to multiple sequences.

Similarity studies: Sequence comparison is one of the most powerful techniques for understanding the organization and function of biological systems. There is continuing need for methods to compare sequences both more easily and more thoroughly. Knowledge bases of experience with the meaning and interpretation of results will also be needed. A variety of methods are used in the laboratory. One of the most powerful of these uses the Cray XMP to search for regions of local homology with the thorough program, SEQHP, or the more rapid SEQFP/SEQFT programs of Kanehisa, and then to uses the SEQDP program to assess the non-randomness of the matches. These have now been combined and modified to produce output suitable for direct input into relational database programs such as FRAMIS or INGRES. Versions were adapted for use in UNIX-based operating systems permitting the use of universally available commands such as "awk", "join", "sort", "sed" and others to accomplish similar results as proprietary relational database programs. Low homologies can not be related unambiguously to common evolutionary background, and rather may reflect common structural motifs, or coincidence. Instances of relationships that are obvious in 3-D structure but are difficult by homology (e.g. the two halves of the enzyme rhodanese), may serve as test cases for methods to assess significance. Instances continue to arise where the full dynamic programs and fast hexical based methods differ significantly in results.

An extension of this concept is to use similarities between new sequences and known crystallographic or other 3-D structural data to permit the generation of a molecular model for a protein that can predict and be challenged by experimental data. Plausible models have been generated for perforin,

apo-lipoproteins, portions of reverse transcriptase and portion of CD-4 antigens using similarity search (DFASTP, SEQFDP, SEQHDP) molecular mechanical (CHARMM, AMBER) and molecular graphics programs (MOGLI, MIDAS, GEMM). In some cases these results are stimulating new insight into the functions of proteins derived from gene sequencing.

Detection of potential RNA secondary structure: RNA and DNA structure are of increasing concern as the innate catalytic activity, and other functional properties of polynucleotides are recognized and more experience with protein-nucleic acid interactions is accumulated. Evidence from simple systems suggest that primary sequence determines 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.

Regions of significantly stable structures are found to correlate with the tat-regulating region already shown by others, the rev-responsive region (Melvin, Cullen, Le, Maizel) and the frame-shift sites in the gag-pol and other regions of retroviruses. These studies have been extended to include other members of the HIV-related family and are being applied to correlations of other regions of the genomes for possible structural features.

Publications:

Chen J, Barber AM, Pedersen J, Brandt-Rauf PW, Carucci J, Murphy RB, Pincus MR. Comparative x-ray crystallographic evidence for a beta-bend conformation as the active structure for peptide T in T4 receptor recognition. *J Protein Chem* 1989;8:87-100.

Hanly SM, Rimsky LT, Malim MH, Kim JH, Hauber J, Dudon MD, Le S-Y, Maizel JV Jr, Cullen BR, Greene WC. Comparative analysis of the HTLV-1 Rex and HIV-1 Rev trans-regulatory proteins and their RNA response elements. *Genes & Dev* 1989;3:1534-1544.

Le S-Y, Maizel JV Jr. A method for assessing the statistical significance of RNA folding. *J Theoretical Biol* 1989;138:495-510.

Malim MH, Bohnlein S, Fenrick R, Le S-Y, Maizel JV Jr, Cullen BR. Functional comparison of the rev trans-activators encoded by different primate immunodeficiency virus species. *PNAS* 1989;89:8222-8226.

- Nakata K, Maizel JV Jr. Prediction of operator-binding protein by discriminant analysis. *Gene Anal Tech* 1989;6:111-119.
- Barber AM, Hizi A, Maizel JV Jr, Hughes SH. HIV-1 Reverse transcriptase: structure predictions for the polymerase domain. *Aids Research and Human Retroviruses* 1990;6:1061-1071.
- Barber AM, Maizel JV Jr. SequenceEditingAligner: A multiple sequence editor and aligner. *Gene Anal Tech* 1990;7:39-45.
- Chen J-H, Le S-Y, Shapiro B, Currey KM, Maizel JV Jr. A computational procedure for assessing the significance of RNA secondary structure. *Computer Appl in the Bios* 1990;6:7-18.
- Le S-Y, Chen J-H, Maizel JV Jr. Efficient searches for unusual folding regions in RNA sequences. In: Sarma RH, Sarma MH, eds. *Proceedings of the 6th Conversations in Biomolecular Stereodynamics*. New York: Adenine Press, 1990;1:127-136.
- Le S-Y, Malim MH, Cullen BR, Maizel JV Jr. A highly conserved RNA folding region adjacent to the cleavage site of OMP/TMP in the envelope gene of primate immunodeficiency viruses. *Nucleic Acids Res* 1990;18:1613-1623.
- Le S-Y, Nussinov R, Maizel JV Jr. Studies of local stability in histone, U-SnRNA and globin precursor mRNAs around transcription termination sites. *Computers and Mathematics with Appl* 1990;20:49-56.
- Peitsch MC, Amiguet P, Guy R, Brunner J, Maizel JV Jr, Tschopp J. Localization and molecular modelling of the membrane-inserted domain of the ninth component of human complement and perforin. *Mol Immunol* 1990;27:589-602.
- Tiley LS, Brown PH, Le S-Y, Maizel JV Jr, Clements JE, Cullen BR. Visna virus encodes a posttranscriptional regulator of viral structural gene-expression. *PNAS* 1990;87:7497-7501.
- Le SY, Chen J-H, Maizel JV Jr. Detection of unusual RNA folding regions in HIV and SIV sequences. *Computer Appl in the Bio* 1991;7:51-55.
- Le S-Y, Nussinov R, Maizel JV Jr. 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		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 CB 08381-08 LMMB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Computer Aided Two-Dimensional Electrophoretic Gel Analysis (GELLAB)		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Peter F. Lemkin, Ph.D.		Computer Specialist IPS, LMMB, NCI
<u>Other Professional Personnel:</u>		
Robert W. Ashmore		Scientific Applications Analyst, PRI/FCRDC
COOPERATING UNITS (if any)		
Dr. E. Lester (U. TN. Ctr. Health Serv.); Dr. J. Myrick (CDC/Atlanta); Dr. P. Rogan (Hershey Med. Sch.); Dr. M. Alley (DTP/NCI/FCRDC); Dr. R. Levenson (Duke Med. Sch.); Dr. P. Sonderegger, T. Hale, (Biochem. Inst., Un. Zurich); (cont.)		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Image Processing Section		
INSTITUTE AND LOCATION Frederick Cancer Research and Development Center, Frederick, MD 21702-1201		
TOTAL MAN-YEARS: 1.1	PROFESSIONAL: 1.1	OTHER: 0.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors B		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>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. It runs on UNIX workstations and the CONVEX supercomputer using X-windows for portable interactive graphics. Recently, remote collaborative multimedia image-conferencing has been added to GELLAB-II with possible use in other fields of research.</p> <p>Substantive applications include: assess stability of long term tumor cell lines (Alley); 2D DNA gels for identifying differentially expressed genes (Rogan and Strathern); cadmium toxicity in urine (Myrick); analyzing sets of lymphocyte gels and long term cell lines from human hematological malignancies to identify potential marker proteins for possible microsequencing (Lester); cerebral endothelial cell morphology control (Bauer and Amberger); transcription control in prostrate cell lines (Getzenberg). GELLAB-II has been exported to the U. of Zurich (Sonderegger and Hale), CDC/Atlanta (Myrick), U. Norway (Krekling). Additional changes to help continued collaboration and exporting of enhanced versions of GELLAB has been actively pursued.</p>		

Cooperating Units (Continued):

Drs. H.Bauer and A. Amberger, (Austrian Acad Wissurschufllen, Salzberg); Dr. T. Krekling (Agr. U. Norway).

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 improving the user interface for GELLAB-II that runs on UNIX workstations and the CONVEX supercomputers.

The GELLAB-II programs (about 170,000 lines of code) have continued to be fine tuned to simplify the user interface and make more use of X-windows graphical interface. Additional tests and analyses were added to the composite gel database program CGELP2 to compute and display multiple class scatter-plots and display of protein-concentration expression-profiles for sets of spots in the database.

A high resolution Molecular Dynamics 300 laser densitometer was integrated into the GELLAB-II system over using Ethernet and PC-NSF letting us scan stained gels or autoradiographs directly onto the disks of our SUN network for later analysis.

This year, Robert Ashmore (PRI/FCRDC) brought the automatic spot pairing AUTOPAIR program (to completely automatically pair spots between two gels) to a working stage. The semi-automatic method was compared with the new method. Additional effort was made in optimizing it using profile analysis to decrease the computation times. Additional analysis was added to more easily integrate it with the CGELP2 database program.

We have modified the segmentation and quantitation program SG2GII to be able to handle giant gels (35x40cm) on about 4096 square pixels (Levenson). The huge amount of data in these images required the re-introduction of a paging algorithm previously used in GELLAB-I, but taken out to improve efficiency when it was ported to GELLAB-II. In addition, the pager was redesigned and optimized to improve performance. The current segmenter code can be compiled to use the paging algorithm or not (the latter is more efficient where enough computer memory is available).

Additional image processing filters were added to the SG2GII spot segmentation and quantitation program to better analyze spots in the 2D DNA gels we are starting to analyze (Rogan). These gels are produced by successive application of restriction enzymes selectively sensitive to methylation. The N-pixel spacing Busse Laplacian filter of the original SG2GII was modified to use averaged pixels giving a major improvement in noise immunity detecting a wide density range of fuzzy spots in highly

textured Southern blots. GELLAB-II was and is being modified as required for use with these gels and for building databases for identifying differentially expressed genes. The developing 2D DNA gel preparation method was modified to take the later image analysis into account.

The reference book on GELLAB-II method and usage has been expanded and updated. It is intended to be used primarily as a reference for users of the GELLAB-II system.

Additional effort was made on the new GELLAB-II X-windows X11 library, libX11IPS. This is helping enforce a consistent windowing user interface on the GELLAB-II software. This then makes it easier to learn GELLAB.

Xpix is a menu driven image processing system for X-windows that is used by many of the GELLAB-II programs to display gel images or maps of spots in gel databases. Work has continued on a the X11 X-windows version of Xpix called Xpix11 using the libX11IPS library.

Research has begun on remote collaboration of 2D gel images using workstations connected via national and international networks. A demonstration program, Xconf, has been written (reported at the 1991 Int. Electrophoresis Soc. meeting). Xconf permits image-conferencing of remote groups of up to 30 collaborators who are network-connected with X-windows workstations. Conferees may interactively point to and discuss image data with actions visible to everyone in the conference. The initial design is for 2D gels, but the concepts and conferencing system should be useful for a wide variety of biological domains both in research and in clinical situations.

Collaborations:

Many of the of collaborations are continuing and some new ones were started: Mike Alley (DTP/NCI/FCRDC) continues investigating the use of 2D gels in testing the stability of cell lines in NCI/FCRDC anti-cancer drug screening program and also for possibly developing sets of marker proteins (also in a joint collaboration with Eric Lester). Eric Lester (U. Tenn.) continues the collaboration on adult human leukemias correlated with patient data and identifying key proteins. Drs. Hans Bauer and Albert Ambrose (Austrian Acad. Sci.) have continued the study of the blood-brain barrier using brain capillary epithelial cells. Robert Getzenberg (J. Hopkins U.) has been studying the alteration of nuclear matrix proteins and its determination of tissue specific gene expression. Peter Rogan (Hershey Med. Sch.) is using of GELLAB-II help analyze new 2D DNA gels based on restriction enzymes to be used for monitoring active transcription sites in yeast and other systems. The IPS is developing extensions to GELLAB-II to handle this new type of data.

We have exported GELLAB-II to: Biochemistry Institute at the Univ. of Zurich (Sonderregger, Hale); Dr. James Myrick (CDC/Atlanta) who is using it with his BioImage system; and the laboratory of Dr. Trygrv Krekling (Agr. Univ. Norway) is collaborating on enhancements.

PROJECT DESCRIPTION

Major Findings:

A highly integrated system for the analysis of RNA secondary and tertiary structure has been developed and is evolving on a SYMBOLICS 3675 and SUN computer (Shapiro, in preparation). 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, a CONVEX, VAXES and the CRAY. These algorithms are invoked from the SYMBOLICS or 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 (Shapiro, Zhang). The ability also exists to search for specific structural elements that are a function of global or semi-global structure, base pairing, local energies as well as sequence. The ability exists to graphically display structures that are generated. One may then interact with the display to get at various local structural elements. The structure may be labeled in different ways so that the important area of current interest may be viewed. Structures may be compared analytically, for example using a Boltzman distribution as well as visually. The system is in the process of being ported to SUN workstations (W. Kasprzak) thus allowing broader access to the research community. The SUN version of the system, which is currently undergoing testing 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.

New algorithms and methodologies have been developed that permit the clustering, searching for, and examining in detail of multiple RNA secondary structures from a structural as well as sequence standpoint. The object is to find structural similarities that could be correlated with functional sites. This includes the examination of how mutations affect structure and function in RNA. 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.

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 (with E. Dayton, A. Dayton, D. Konings, D. Powell, L. Butini and J. Maizel).

Computer and biological experiments have been pursued in studying the lambda TR2 terminator. 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 S-W Chang-Cheng, E. Lynch, K. Leason, D. Court and D. Friedman).

A study of potential RNA pseudoknots just downstream from known and suspected retroviral frameshift sites has been done. 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. This work was done in collaboration with S-Y Le, J-H Chen, R. Nussinov and J. Maizel.

Work has been almost completed on a methodology to predict pseudoknots from RNA secondary structures. This is based on the premise that most pseudoknotted structures that 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 D. Konings).

Work has continued in collaboration with K. Currey on the structural effects of virulent and non-virulent polio strains I, II and III. This has led to the development of new algorithms to permit consideration of RNA tertiary structure, specifically loop-loop interactions which includes the possibility of pseudo-knots, in regard to polio's functional sites. Experimental

mutational data is being included (Trono et. al., 1989) to better relate the structural aspects to functional regions of the 5' noncoding regions. Initial observations suggest that secondary structure and possibly tertiary structure in the form of neighboring stems with loop-loop interactions may correlate with ribosomal binding sites.

A phase of the work utilizing a new tree comparison algorithm to do flexible and accurate comparisons of RNA secondary structures has been completed (B. Shapiro, K. Zhang). This included the ability to determine precisely where differences reside between two structures and to place a measure on these differences. This is analogous to the sequence homology algorithms commonly used but here structures are being compared. The algorithm permits several levels of abstraction in comparing RNA structures. The structures may be compared based upon a loose morphologic description of their shape. A more refined comparison is possible allowing for the sizes of the individual structures to be included. Ultimately sequence information may be included. The algorithm mentioned here has been included in the above described system to get an even better handle on comparative structures.

Work has been done on a new algorithm that speeds up the dynamic programming version of the planar RNA folding algorithm. The algorithm computes places where it can skip over various vertices because an improvement in energy would not be possible if the intervening vertices were considered. This reduces the number of locations that must be examined in the folding matrix. This work has been done in collaboration with R. Nussinov, S-Y Le, and J. Maizel.

Exploratory work has continued in an attempt to visualize the rev responsive element of HIV-1 using a scanning tunneling microscope. This microscope has the potential resolution of less than 1 angstrom in the horizontal direction and less than 1 angstrom in the vertical direction. It is hoped that the secondary and tertiary structures of the molecule will be visualized with this technique. Samples of the RNA REV responsive elements of HIV-1 were sent to Melvin Pomerantz who made some scans with his tunneling microscope. The resultant images were then processed within our laboratory to image enhance the results. First results look encouraging in that we are seeing objects that appear to be the right dimensions and appear to be double helical. This has encouraged us to prepare more samples and some different techniques to further enhance the results. This work is being done in collaboration with M. Pomerantz, A. Dayton, and J. Maizel.

Publications:

Shapiro BA, Zhang K. Comparing multiple RNA secondary structures using tree comparisons. CABIOS, 1990:6:4:309-318.

Nussinov R, Shapiro B, Le S, Maizel J. Speeding up the dynamic algorithm for planar RNA folding. Mathematical Biosciences, 1990:100:33-47.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08386-05 LMMB

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Heterogeneity of β (1-4)galactosyltransferase Transcripts

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

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Research Chemist

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Arni Masibay, Ph.D.

IRTA Fellow

LMMB, NCI

COOPERATING UNITS (if any)

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TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

Lactating mammary gland from various species expresses two major mRNAs for β 1,4galactosyltransferase; one 4 kb and other 2.5 kb. The 4 kb mRNA has a 2.7 kb long 3' noncoding sequence. The 2.5 kb mRNA is short 1.5 kb of the 3' end sequence of 4 kb mRNA and is generated by utilizing the polyadenylation signal present within the 3' noncoding sequence of the 4 kb mRNA. Primer extension analysis of the mRNA from various tissues show that the β 1,4galactosyltransferase mRNA in the lactating mammary glad is about 230 bases shorter at the 5' end compared to the mRNA produced in kidney and embryonal trachea cells. The mammary glad mRNA has only the second in frame ATG codon compared to the mRNA from kidney and embryonal trachea and thus codes for a protein that lacks the first thirteen residues but still contains the putitative membrane-anchoring domain.

In the lactating mammary gland in addition to the two major mRNA species other β 1,4galactosyltransferase sequence-related transcripts were identified. These sequence-related transcripts are generated by utilizing the polyadenylation signals within the introns for poly A⁺ synthesis and by partial processing of the precursor RNA. Sequence analyses of many of these transcripts showed that none of these poly A⁺ containing RNAs have any long open reading frame within the sequences which are not present in the 4 kb or 2.5 kb mRNAs. The significance of very high proportion of these aborted molecules in the lactating mammary gland remains to be determined.

Project DescriptionMajor Findings:Molecular Heterogeneity of $\beta(1-4)$ Galtransferase transcripts:

Northern Analysis of the RNA from various tissues:

Northern analyses of the RNA from various tissues showed that the lactating mammary gland compared to other tissues produces besides the 4 kb long $\beta(1-4)$ Galactosyltransferase mRNA, a 2.5 kb mRNA species. Hybridization of the RNA with the probes generated from 4 kb mRNA showed that the 2.5 kb mRNA lacks 3' end region of the 4 kb mRNA. Sequence analyses of the 3' end of the cDNA clones corresponding to these mRNAs showed that the 2.5 kb mRNA is generated by utilizing the polyadenylation signal present in the 3' noncoding sequence of the 4 kb mRNA.

Primer Extension Analysis of the mRNA from various tissues:

Primer extension analyses of the total RNA and poly(A)⁺RNA from lactating mammary gland, kidney (MDBK) and embryonal trachea (EBTr), with a 30-mer oligonucleotide primer showed that the major extension product of the lactating mammary gland RNA is much shorter than the product obtained from other RNAs. Detailed analyses of the extended primer product on the sequencing gels show that the transcript in the mammary gland has a short 5' noncoding sequence that lacks the first ATG codon and thus the transcript encodes a protein that is missing the first 13 amino acids. Gel analyses of the primer extension products from other tissues show that there are two major transcripts that have 221 and 238 nucleotide long 5' noncoding sequence, respectively. Each transcript contains two inframe ATG, translational initiation codons, that are 36 nucleotides apart. These transcripts will encode a protein that has 13 amino acids more at the amino-terminal end compared to the protein encoded by the transcript made in the lactating mammary gland.

Analyses of $\beta(1-4)$ galactosyltransferase mRNA related aberrant poly A⁺RNA molecules:

Nucleotide sequence analyses of many cDNA clones showed that there are poly(A)⁺ containing molecules whose partial sequences correspond to the $\beta(1-4)$ galactosyltransferase mRNA sequence. Sequence comparison of these molecules show that they represent partially processed precursor RNA molecules that have utilized the polyadenylation signals present in the introns generating the poly A⁺ containing RNA. None of the sequences present in these related molecules that are absent from the functional $\beta(1-4)$ galactosyltransferase mRNA show any open reading frame. In the lactating mammary gland function of such a high number of partially processed poly A⁺ containing RNA molecules related to galactosyltransferase mRNA remains unknown.

Expression and regulation of β 1-4galactosyltransferase during 3T3 cell growth:

β 1-4galactosyltransferase enzyme is localized in the trans Golgi where glycoproteins get galactosylated. It has also been found at the cell surface where it acts as cell-adhesion molecule, mediating intercellular adhesion by binding to terminal N-acetylglucosamine residues on lactosaminoglycans substrates, there by bridging cells together, and as a high affinity receptor for the laminin long arm. Upon growth-stimulation of quiescent 3T3 cells expression of several genes is sequentially induced. We monitored the level of galactosyltransferase mRNA and protein during the 3T3 cell-cycle. Our results show that:

1) The β 1-4galactosyltransferase mRNA increases 4 to 5 fold after 2 h of serum-stimulation of quiescent 3T3 cells. The mRNA induction parallels the expression of a set of "immediate early" and "early" genes like c-fos, steroid receptors, c-jun, c-myc, JE, Vimentin, gamma and beta actin, etc., in that the β 1-4galactosyltransferase mRNA expression occurs early during the cell's transit from G_0 to S, and does not require prior protein biosynthesis. Protein biosynthesis inhibitors like cycloheximide and anisomycin superinduce β 1-4galactosyltransferase mRNA expression.

2) The β 1-4galactosyltransferase protein levels and activity increase on about the same time scale as the mRNA. The observed increase in the galactosyltransferase activity is associated both with the plasma membrane and Golgi fraction. In contrast to the increase in Golgi pool during differentiation of embryonal carcinoma cells, it is both the plasma membrane and Golgi pool of β 1-4galactosyltransferase that increases during the transit of 3T3 cells from G_0 to S.

Our results suggest that β 1-4galactosyltransferase may have additional roles besides its known function in the Golgi apparatus. However, the difference(s) between the cell surface and Golgi bound galactosyltransferase, the mechanism of the differential regulation of the two forms of galactosyltransferases and the role of cell surface galactosyltransferase during cell growth remains to be determined.

Publications:

Boeggeman E, Masibay AS, Qasba PK, Sreevalsan T. Identification and partial characterization of genes that are transactivated by different pathways in quiescent mouse cells stimulated with serum. *J Cell Phys* 1990;145:286-294.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08387-04 LMMB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Nanoanatomy & Topochemistry of the Outer and Cytoplasmic Surfaces of Biomembranes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Sect. LMMB, NCI		
<u>Other Professional Personnel:</u>		
Ru-Long Shen, M.D.	Visiting Fellow	LMMB, NCI
Wang-Ying Hou, M.D.	Visiting Fellow	LMMB, NCI
Paola Sirigu, M.D., Ph.D.	Guest Researcher	LMMB, NCI
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Eliana Munoz, M.T.	Research Associate	PRI/FCRDC
Cristina Risco, Ph.D.	Post Doctoral Fellow, Min. of Ed., Spain	(G.R. PRI/FCRDC)
COOPERATING UNITS (if any) Dr. P.F.P Pimenta, McArthur Foundation, FAES, NIH; Drs. L. Menendez-Arias and S. Oroszlan, Lab. of Molec. Viro. and Carc. (NCI-FCRDC); Drs. C. Romero and M.A. Bosch, Dept. of Biochem. and Molec. Biol., I. Universidad Complutense (continued)		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Membrane Biology Section		
INSTITUTE AND LOCATION Frederick Cancer Research and Development Center, Frederick, MD 21701		
TOTAL MAN-YEARS: 6.0	PROFESSIONAL: 6.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) We investigate structural and cytochemical areas of membrane biology. We use a series of methods developed in our laboratory, in particular: a) <u>fracture-flip</u> (to obtain images of the outer and cytoplasmic surfaces of plasma membranes and maps of the distribution of surface receptors and antigens by immunogold labelling; b) <u>label-fracture</u> and replica staining label-fracture (to relate the inner structure of the membrane to its surface topochemistry); and c) <u>simulcast</u> (to compare nanoanatomical views of membrane surfaces with freeze-fracture aspects and cytochemical maps in one single membrane). We investigate the localization of cytoplasmic and of membrane-associated oncogene products; the ultrastructural pathology of human parasites; the membrane dynamics of CD4 and of HLA antigens; cellular basis of bacterial infections; and ultrastructural and cytochemical aspects of the maturation of mouse mammary tumor virus and of nerve cells in culture. In addition, we continue our research on the structure of the zonula occludens, to determine transcellular continuity of the exoplasmic leaflets of the plasma membrane. Project numbers Z01 08374-06 and Z01 08376 are now combined with this project.		

Cooperating Units (Continued):

de Madrid, Spain; Drs. A. Guidotti and H. Manev (Fidia-Georgetown Inst. for the Neurosciences, Georgetown Univ., Wash., D.C.); Drs. R. Anadon and M.I. Rodriguez-Moldes (Dept. of Funda. Biol., Univ. of Santiago de Compostela, Spain); Drs. M.R. Torrissi, A. Pavan, P. Mancini, (Inst. of Gen. Path., U. Rome Sch. Med., Rome, Italy); Drs. D. Nishioka, R. Ward, (Dept. Bio., Georgetown U.); Drs. W. deSousa, W.M. Kattenbach (Inst. of Biophys., Fed.Univ. Rio de Janeiro, Brasil); Drs. P.S. Doyle, J.C. Engel, D.J.T. Nash (Lab. Parasit. Dis., NIAID, NIH); Dr. F.W. Kan (Dept. Anat., Univ. of Montreal, Montreal, Canada); Dr. A.P. Aguas and Manuel T. Silva (Center of Exp. Cytology, Porto, Portugal); Drs. R. Zhou, G.F. Vande Woude, P. Sutrave, and S.H. Hughes, ABL, NCI-FCRDC; Dr. J.F. Mousa-Nunes, Port. Cancer Inst., Lisbon, Portugal.

PROJECT DESCRIPTION

Major Findings:

A. Localization of oncogene products (Shen, Zhou, Vande Woude, Hou, Shih, Pinto da Silva). We used immunogold labelling methods to demonstrate the colocalization of the mos oncogene present and of tubulin in X₃ cells. It is known that the disruption of microtubules with TBZ prevents the activation of cdc 2 and the degradation of cyclin protein. The interaction of mos and tubulin could affect microtubule/ or centrosome functions that, in turn, regulate cdc2/cyclin function. We are now trying to define the relationships between mos oncogene product, tubulin and cdc2 in *Xenopus* eggs. (There are high concentrations of mos product in unfertilized eggs; low concentrations in fertilized eggs). The work on the localization of the product of ski oncogene (a nuclear oncogene that can cause the selective growth of the skeletal muscle cell) is now being started on transformed chicken fibroblasts. The work will be extended to pig muscle cells and to mitotic cells to relate the localization of ski with that of other proteins. In another project we were able to use a modification of fracture-flip (a method developed in our laboratory) that involves detergent/enzyme action to expose and label the inner surfaces of the plasma membrane of fibroblasts in culture. We have now obtained the first in situ three-dimensional views of the membrane skeleton in these cells and have now started working on the application of immunogold labelling to determine the distribution of p21, a ras oncogene product. We should then extend our work to other oncogene products associated with cytoplasmic surfaces of the plasma membrane.

B. Pathogenic and non-pathogenic states of human parasites. This part of the project uses cells that, contrary to most cells in vertebrates, show high domain differentiation. Thus, in addition to the enormous importance of these cells as agents of diseases that affect a large segment of the world's population (n.b. some of these parasitic infections - e.g. *Giardia*, *Amoeba*, are becoming of increasing relevance within the Continental U.S.). These cells afford us a unique opportunity in the development of our methods: their membranes contain a variety of ultrastructurally clear structural and cytochemical markers that have been of significant help to us in the

development of new ultrastructural and immunocytochemical schedules. This work has resulted in manuscripts already submitted for publication reporting the 1st views of the inner surface of the plasma membrane in *Leishmania*, (Hou, Pimenta, Pinto da Silva), the effect of long term culture of axenic amastigotes at 37°C (Doyle, Engel, Pimenta, Pinto da Silva) and the nanoanatomical and topochemical demonstration of surface variants in *Giardia lamblia* (Pimenta, Kattenbach, de Souza, Pinto da Silva, Nash).

C. Simulcast (Shen, Pinto da Silva). We developed this new method to reveal the inner structure nanoanatomy and topochemistry in one single membrane. The new method allows in one single electron microscopical image to obtain conventional freeze-fracture views [Protoplasmic (P) fracture faces/Exoplasmic (E) fracture faces], fracture flip surfaces [views of either the outer or inner surface where structures as small as 5nm can be resolved], and immunogold labelling of components at either surface.

D. Surface dynamics of HLA and CD4 antigens on human lymphocytes (Pavan, Mancini, Torrisi, Pinto da Silva). This year we have concluded the first part of a project to investigate the distribution of HLA, DC3 and CD4 antigen, and their dynamics during capping. To this end we used label-fracture and fracture-flip to provide what are truly remarkable views of the process of capping. Among other findings we showed that capping of one set of antigens must co-involve the emigration of other membrane proteins from the capping pole of the lymphocyte. In addition, and contrary to previous belief, we showed that HLA and CD4 antigens are represented in freeze-fracture images by intramembrane particles. In future experiments we will attempt to find experimental schedules to reveal the inner surface of the plasma membrane in lymphocytes. Access to the inner surface of these cells is needed to define important transmembrane linkage during the process of migration of surface antigens, in particular the participation of components of the skeleton at the inner surface of the membrane. In other work (Shen, Nishioka, Ward, Pinto da Silva) we applied label-fracture, fracture-flip and replica-staining label-fracture, to the study of the comparative distribution of surface antigens in sperm cells labelled with colloidal gold/monoclonal antibody conjugates.

E. Cellular and molecular basis of bacterial infections (Shen, Aguas, Teixeira da Silva, Pinto da Silva). One project involves collaboration with one of the foremost laboratories in the study of the ultrastructure pathology of lepra. The work involves conventional freeze-fracture exploration of infected cells as well as the obtention of the first high resolution surface views of these bacteria. The second project (Risco, Romero, Bosch, Pinto da Silva) we study the cellular and molecular mechanism of septicemia (bacteremia) inducing bacterial endotoxins. This is an important (but, surprisingly, hardly investigated at the NIH) area since more than 200,000 (mostly fatal) cases of septicemia are registered every year in the U.S. This despite the availability of potent antibiotics. To date, most of the work involves the use of water insoluble LPS aggregates. Instead we are using monodisperse LPS as carried in the serum of infected animals to investigate the pathobiology of this endotoxin. The project involves ultrastructural and cytochemical approaches to study the interaction of LPS in vivo with alveolar macrophages as well as erythrocytes.

F. Cytochemical aspects of the maturation of mouse mammary tumor virus (Risco, Menendez-Arias, Oroszlan). In these experiments we have started a collaborative project to investigate proteins related to viral maturation, in particular related to viral proteases that could be connected with the transformation of immature A particles. It will be possible to study the presence or absence of specific proteins in distinct steps of the life of the virus and its interaction with plasma membranes.

G. Nanoanatomy and topochemistry of the phase membranes of nerve cells (Caruncho, Guidotti, Manev, Anadon, Rodriguez-Moldes). This project continues the initial exploration by our previous Visiting Fellow, C. Andersson-Forsman (manuscript in preparation) and aims at study of the distribution of Glutamate receptors in cultured cerebellar granule cells using specific antibodies to investigate the possible movement of protein kinase C from the cytosol onto the inner surface of the plasma membrane distribution and signal transduction of GABA receptors in transformed kidney cells that were transfected with one plasmid containing the gene for the GABA receptor.

H. Intercellular junctions (Shen, Pinto da Silva). We have used freeze-fracture, fracture-flip, freeze-etching and thin section electron microscopy to demonstrate the transcellular continuity of exoplasmic apical and of basolateral halves in epithelia joined by the Zonula.

J. Invited reviews (Pinto da Silva, see references). I have continued to correspond to demands for review and publicize the recent work in our laboratory. In the past decade our laboratory has become the world leader in the development of new immunocytochemical and nanoanatomical methods to investigate the surfaces of biological membranes.

Publications:

Pavan A, Mancini P, Cirone M, Frati L, Torrisi MR, Pinto da Silva P. The capping of HLA antigens in human lymphocytes as followed by immunogold label-fracture. *J Histochem Cytochem* 1989;37:1489-96.

Pinto da Silva P. Freeze-fracture images: 1967-1971. In: Hui SW, ed. *Freeze-Fracture Studies of Membranes*. Boca Raton, FL: CRC Press 1989;1-9.

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Torrisi MR, Mancini P, Pinto da Silva P. Cytochemical access to plasma and intracellular membranes of freeze-fractured hepatocytes and salivary gland cells. In: Motta P, Riva A, eds. *Microanatomy of cells and tissues*. Kluwer Academic Publishers 1989;177-82.

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Pavan A, Mancini P, Lucania G, Frati L, Torrisi MR, Pinto da Silva P. High resolution surface views of human lymphocytes during capping of CD4 and HLA antigens revealed by immunogold fracture-flip. J Cell Sci 1990;96:151-57.

Shen RL, Pinto da Silva P. Simulcast: contiguous views of fracture faces and membrane surfaces in a single cell. Eur J Cell Biol 1990;53:122-130.

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 and Dev 1991. In press.

Renping Z, Shen RL, Pinto da Silva P, Vande Woude GF. Further characterization of pp39^{mcs} association and phosphorylation of tubulin. Cell Growth and Diff. 1991. In press.

Doyle PS, Engel JC, Pimenta PFP, Pinto da Silva P, Dwyer DJ. Leishmania donovani: long term culture of axenic amastigotes at 37°C. 1991 J Exp Parasitol In press.

PROJECT DESCRIPTION

Objective:To determine "functional" regions or domains of β 1,4galactosyltransferase.

Using the methodology of the recombinant DNA technology, we are developing general methods to produce the normal and altered proteins. Mammalian and prokaryotic expression vectors are engineered in such a way that the same vector can be used to produce protein in quantities enough to test its function, and as well to produce the engineered protein in large quantities for determining various physical properties and perform X-ray crystallographic studies.

Major Findings:Preparation of deletion constructs of bovine β 1,4galactosyltransferase cDNA in eukaryotic vector and their expression in COS-7 cells:

A full-length bovine galactosyltransferase 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 promoter. 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 α -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 β 1,4galactosyltransferase expression vector, pLsGT. We have deleted the DNA sequences which code for the potential membrane anchoring domain of the galactosyltransferase protein and the first amino-terminal 70 residues which are 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 β -galactosidase coding vector produced the specific proteins. The results suggest that the membrane-anchoring domain of β 1,4galactosyltransferase has a role in mammalian cells either to stabilize the protein or to prevent it from inactivation.

Preparation of deletion constructs of bovine β 1,4galactosyltransferase cDNA in prokaryotic vector and their expression in E. coli:

Bovine β 1,4galactosyltransferase was expressed as fusion protein with the glutathione S-transferase in Escherichia coli using the novel expression system pGEX 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-3T 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 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 β 1-4galactosyltransferase.

Both forms of β 1-4galactosyltransferase protein produced in *Escherichia coli*, the full-length 402 residue long protein that contains the amino-terminal membrane-anchoring domain and the 332 residue-long secreted form of the protein that lacks the first 70 residues of the protein, are enzymatically active. This is in contrast to the results observed in mammalian cells where the secreted form of the protein within the cell for as yet unknown reasons is not active.

The amino acid requirements for the binding of Ca^{2+} in alphalactalbumin:

Alpha-lactalbumin, a metalloprotein that binds Ca^{2+} , modifies the enzymatic activity of β 1-4galactosyltransferase 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 β 1-4 glycosidic linkage in polysaccharides. Lysozymes from different species, with the exception of horse, dog and pigeon, do not bind Ca^{2+} . The 3-D structure of these two homologous proteins, alpha-lactalbumin and lysozyme, as well as their gene structure, are very similar. The alpha-carbon atom backbone of the Ca^{2+} -binding loop of alpha-lactalbumin is very similar to the corresponding region of c-lysozymes but different from the EF-structure of other Ca^{2+} -binding proteins. The high resolution X-ray structure analysis of baboon alpha-lactalbumin shows that Ca^{2+} -binding site is located in a bend or elbow formed from 10-residues. Five of these are liganded to Ca^{2+} ion. Of these three are aspartic acids that interact with the bound Ca^{2+} 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 Ca^{2+} -binding lysozyme 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 Ca^{2+} -binding lysozyme. 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 Ca^{2+} via another water molecule. Also the conserved hydrophobic residues may be involved in controlling the hydration sphere of the bound Ca^{2+} ion which results in more stable cation complex. Genetic manipulation of the alpha-lactalbumin cDNA clone is directed towards testing this hypothesis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08392-03 LMMB

PERIOD COVERED
October 1, 1990 to September 30, 1991TITLE 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, laboratory, and institute affiliation)

John N. Weinstein, M.D., Ph.D., Chief, Theoretical Immunology
Section LMMB, NCIOther Professional Personnel:Janos Szebeni, M.D. Visiting Scientist LMMB, NCI
Vellarkad N. Viswanadhan, Ph.D. Visiting Scientist 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 MAN-YEARS:

2.4

PROFESSIONAL:

1.4.

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

We recently 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:

- 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.
- 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.
- Molecular biology: Methods based on polymerase chain reaction are being used to clone the nucleoside transport protein, a major target for DPM.
- 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-weighting technique to generate updated weights; (iii) repeat regression with the updated weights; (iv) iterate the previous two steps; (v) use a combination of normal theory and Monte Carlo techniques to determine confidence limits on the fitting parameters; (vi) construct a set of derived parameters that express various aspects of synergy, potentiation, and antagonism; (vii) calculate confidence limits on those constructed parameters; (viii) produce graphical displays of the error model, the residuals of each fit, and the contours of equipotent drug effect. 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.

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).

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Viswanadhan VN, Weinstein JN, Elwood PC. Secondary structure of the human membrane-associated folate binding protein using a joint prediction approach. *J Biol Structure and Dynamics* 1990; 7:985-1001.

Weinstein JN, Schinazi RF, Wahl SM, Bunow B, Gartner S, Popovic M, Wahl LM, Weislow OS, Szebeni J. Synergistic drug combinations in AIDS therapy: Dipyridamole-zidovudine in particular and principles of analysis in general. *Annals NY Acad Sci* 1990;616:367-384.

Patel SS, Szebeni J, Wahl LM, Weinstein JN. Effect of dipyridamole on transport and phosphorylation of dideoxycytidine and cytidine in human monocyte-macrophages. *Biochem Pharmacol* 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 08394-03 LMMB

PERIOD COVERED October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Non-Contiguous Patterns and Functional Domains in DNA

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

Andreas K. Konopka, Ph.D. Visiting Scientist LMMB, NCI

Other Personnel:

John Owens Computer Specialist LMMB, NCI

COOPERATING UNITS (if any)

Dr. R. Micali, Computer Science Lab., MIT; Dr. R. Macgregor, Biophysics, AT&T Bell Laboratories; Dr. A. Sarai, RIKEN Life Sci. Center, Ibaraki, Japan; Dr. T. Jovin, Max-Planck Institute, Goettingen, FRG.

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 MAN-YEARS:

1.75

PROFESSIONAL:

1.0

OTHER:

0.75

CHECK APPROPRIATE BOX(ES)

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

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

The main goal of this project is to provide a consistent methodology for locating putative functional domains in unannotated nucleic acid sequences.

Studies are conducted in two directions:

- 1) Determine by computational experiments, function-associated sequence patterns in large collections of functionally equivalent sequences.
- 2) Design protocols that will help "translate" observed sequence patterns into discriminant functions usable for mapping.

A software for mapping not only genes but also various kinds of non-coding regions resulted from these studies. Detailed methodological guidelines for finding putative functional domains from sequence data are now being worked out.

Project No. Z01 CB 08395-02 LMMB has been converged with this project. The database of illegitimate recombination sites is currently analyzed for the occurrence of significant non-contiguous pattern. A routine to map illegitimate recombination regions in unannotated sequences is now in preparation.

PROJECT DESCRIPTION

Major Findings:

Results obtained in the previous years of this project suggest that local compositional complexity is a good "by content" criterion to approximately locate introns and exons. It can also be used to find genes of functional RNAs (ribosomal and transfer). In order to increase mapping accuracy the method has been enriched in several "by signal" criteria. However, the method is still far from being general. Not only do other methods need to be developed, tested and compared with the existing ones but also a general methodology to determine functional domains (from sequence data) will have to be designed.

On the way to the above mentioned methodology the following tasks have been completed:

- 1) A working classification of sequence (or structure) patterns has been designed (Konopka, manuscript in preparation)
- 2) A general theory of discriminant analysis has been developed and implemented on the test data (Konopka, manuscript in preparation).

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. The above theoretical studies will assist us greatly in creating routines for mapping putative functional domains.

Publications:

Konopka AK. Towards mapping functional domains in indiscriminantly sequenced nucleic acids: A computational approach. In: Sarma R, Sarma M, eds. Human genome initiative and DNA recombination. New York: Adenine Press, 1990:113-125.

Konopka AK, Sarai A. Somatic cell illegitimate recombination via a tetraplex DNA intermediate, In: Sarma R, Sarma M, eds. Human genome initiative and DNA recombination. New York: Adenine Press, 1990:243-251.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		201 CB 08396-03 LMMB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Information Theory in Molecular Biology		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) Thomas D. Schneider, Ph.D. Staff Fellow LMMB, NCI		
Other Personnel:		
Denise Rubens	Research Associate	PRI/FCRDC
R. Michael Stephens	Linganore H.S., Frederick, MD & MIT, Cambridge, MA	
Ian M. Blair	Montgomery Blair H.S.	
Nathan Herman	Frederick H.S., Frederick, MD	
Mark C. Shaner	Frederick H.S., Frederick, MD	
COOPERATING UNITS (if any) John Spouge, NLM (Bethesda); Peter Bassler, DRS, BEIB (Bethesda); Sharlene R. Matten, Dept. of Biochemistry, Univ. of Maryland; William S.A. Brusilow, Wayne St. Univ., Detroit, MI; George Pavlakis, ABL (Frederick); Dhruva K. (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 MAN-YEARS 2.75	PROFESSIONAL: 1.0	OTHER: 1.75
CHECK APPROPRIATE BOXES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		B
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Shannon's measure of information is useful for characterizing the DNA and RNA patterns that define genetic control systems. Thus we may measure the amount of pattern which a ribosome has available to it in the mRNA. In <i>E. coli</i> this is about 11.0 bits per site, where one bit is the choice between two equally likely possibilities. A single measurement is meaningless unless we have something to compare it to. Fortunately, we can calculate the information required to select ribosome binding sites from other mRNA sequences. This is 10.6 bits per site, almost identical to the amount available. Thus the amount of pattern at ribosome binding sites is just about the amount of information needed to find the sites. This is a "working hypothesis": exceptions will either destroy the hypothesis or reveal new phenomena. Several genetic systems now fit the hypothesis, but a few provide striking exceptions which we are actively studying. In particular, the sequences at which bacteriophage T7 bind have twice the information required to locate them. The most likely explanation is that two proteins bind the DNA. The project has three major components: theory, computer analysis and genetic engineering experiments. Computer analysis and theory predict that the T7 promoters and RepA binding sites have anomalies, so we are performing experiments to find out why. 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.		

Cooperating Units (Continued):

Chattoraj and Peter P. Papp, Laboratory of Biochemistry, National Cancer Institute, (Bethesda); Dennis Arvidson, P. Youderian (California Institute of Biological Research, La Jolla, CA) and Gary D. Stormo (University of Colorado, Boulder).

PROJECT DESCRIPTION

Major Findings:

Molecular Machines

The first two papers (level 1 and 2) of the theory of molecular machines have now been published (J. Theor. Biol. 148(1):83-123,125-137, 1991). Several more papers are in preparation. Molecular machines are defined in the first paper as molecules which are capable of making decisions. For example, the EcoRI enzyme can distinguish the sequence 5'GAATTC 3' from the 4095 other 6 base pair long DNA sequences. It is able to do this with a precision that is puzzling workers in the field. The theory of molecular machines explains how EcoRI achieves this feat. In addition, it is a general theory that also applies to DNA hybridization, detection of light by rhodopsin and the mechanism of muscle, so the explanation of precision is important to all biology. In the second paper the ultimate limits of both molecular machines and computers become clear. The limit is known as " $k_B T \ln(2)$ " in the literature, but this is a bit of folklore since it is not an equation, and the units have been consistently neglected by workers in the field. The equation is $E_{min} = k_B T \ln(2) \leq -q/R$, where E_{min} is the minimum energy dissipation of a molecular machine, in joules ($-q$) per bit (R). Surprisingly, it is easily derived from the Second Law of Thermodynamics, a fact that has been forgotten or never realized in the literature. The literature is full of a battle in which " $k_B T \ln(2)$ " is questioned. The authors do not realize that they are objecting to the Second Law, and so by implication are proposing perpetual motion machines! A second surprise is that the equation given above can be derived from the channel capacity formula. It is amazing that such a fundamental relationship has not been clearly understood for the past 40 years. The results define the ultimate limits on small yet powerful computers that may be designed by molecular biologists and engineers.

Sequence Logos

This is a graphical method we (Stephens and Schneider) invented for showing the patterns at binding sites. I now use this method to display all my results, and many people around the world are also starting to use it. The paper announcing the technique was published (Nucl. Acids Res. 18:6097-6100, 1990), and a computer archive was created to make the programs available world-wide (anonymous ftp to ncifcrf.gov in pub/delila). Many groups have obtained the programs for their own use, and I have created logos for several people. Since the publication of the paper last fall, the technique of generating sequence logos was advanced in several ways. A major technical

problem with PostScript graphics was solved and the program was completely rebuilt to make it logical and easy to use. Sequence logos now are in color, which allows subtle variations to be seen with one's peripheral vision. They are also numbered, and have error bars and labels to help interpretation. Examples of color sequence logos will be published in two books: the Pattern Book (C. Pickover, ed) and Visual Recipes: A Scientist's Guide to Visualization (P. R. Keller, ed), a handbook to be published in early 1992. Joe Mack (ABL, Frederick) has just published a sequence logo of the viral integrases, as in HIV, on the cover of the RNA Tumor Viruses Meeting Abstracts.

RepA Project

Information analysis of the P1 plasmid RepA binding sites showed an anomalous information peak in the sequence logo. In collaboration with Dhruba K. Chattoraj and Peter P. Papp, we have synthesized many variations of the RepA binding site. We then selected, cloned and sequenced 100 of those that still bind to RepA. As predicted, the anomalous peak was absent from the experimental sequence logo. The results also fit recently proposed models for the mechanism of plasmid replication. A series of three papers are in preparation, and should be submitted this summer.

Excess Information Content

Nathan Herman, this year's Student Intern Program student from Frederick High School, has been helping me gather binding site sequences for the RepA project. In addition, he discovered a case of triple information content. Ribosome binding sites contain just about the amount of information required to locate them in the genome. We are studying T7 promoters (see above) because the T7 genomic patterns contain twice the information as they should require. Thus Nathan's discovery extends the information theory results in an important way. We are writing this discovery as a small paper.

T7 Project

Denise Rubens has set up the laboratory and we finally have all the media and equipment necessary to sequence variations of bacteriophage T7 promoters. Having solved several mechanical failures and technical problems, she is just beginning to obtain T7 sequences. 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 technique of sequence logos and the results of the RepA project are giving us clues about what to expect and look for in this project.

Perceptron Analysis

I analyzed the *E. coli unc* operon using a neural network technique invented by Gary Stormo and me in 1982. I detected a ribosome binding site in the middle of the *uncB* gene. Sharlene R. Matten and William S.A. Brusilow recognized that this would explain why translation seems to decrease in the middle of *uncB*, a feature that had been puzzling them. They have now shown experimentally that downstream translation is increased if the putative stall sequence is disrupted. This work is being presented at the American Society for Microbiology in Dallas, Texas.

Automated 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 we have submitted it as a short paper.

Splice Junction Analysis

A paper with R. Michael Stephens, described in last year's report, was submitted for review.

AIDS Rresearch

1% (2 days) In collaboration with George Pavlakis, I used an information theory technique (in preparation) to search through the HIV genome for splice junctions. Dr. Pavlakis noted a correlation between the theoretical predictions and cryptic splice junctions.

Publications:

Schneider TD. Theory of molecular machines. I. Channel capacity of molecular machines. *J Theor Biol* 1991;148:83-123.

Schneider TD. Theory of molecular machines. II. Energy dissipation from molecular machines. *J Theor Biol* 1991;148:125-137.

Schneider TD, Stephens RM. Sequence logos: A new way to display consensus sequences. *Nucl Acids Res* 1990;18:6097-6100.

Schneider TD. Genetic patterns as shown by sequence logos. In: Pickover C, ed. *Pattern Book*. Cos Cob, Connecticut: Manning Publications Co, co-publisher IEEE Press, 1991, in press.

Schneider TD. Protein patterns as shown by sequence logos. In: Keller PR, ed. Visual recipes: A Scientist's guide to visualization. 1991, in press.

SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS
NATIONAL CANCER INSTITUTE
1991

The Laboratory of Pathology is responsible for all the diagnostic services in anatomic pathology for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. A fully accredited 4-year residency program in anatomic pathology is provided for 9 residents and 3 fellows. The Laboratory is divided into 9 sections:

Surgical Pathology Section (Dr. Maria J. Merino, Chief)
Pulmonary and Postmortem Section (Dr. William D. Travis, Chief)
Cytopathology Section (Dr. Diane Solomon, Chief)
Ultrastructural Pathology Section (Dr. Maria Tsokos, Chief)
Biochemical Pathology Section (Dr. David D. Roberts, Chief)
Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
Gene Regulation Section (Dr. David L. Levens, Chief)
Office of the Chief (Dr. Lance A. Liotta, Chief)

All sections conduct investigative work and provide research opportunities for the residents. Investigative work completed or in progress is listed by section as follows.

Surgical Pathology Section

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.

Dr. Axiotis has ongoing collaborative studies with the liver unit, involving hepatocellular carcinoma, primary biliary cirrhosis and chronic active hepatitis, and Dr. Gitie Jaffe is actively participating in the study of clonality in parathyroid adenomas.

Pulmonary and Postmortem 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.

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, Pulmonary and Postmortem Section, and the Ultrastructural Pathology Section. Neuropathology diagnostic (patient care) service and teaching (of pathology residents) are provided. Approximately 250 neurosurgical specimens were examined last year. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations, including dementia, pituitary adenomas, PML and gliomas.

Cytopathology Section

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.

Our 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. We have evaluated several monoclonal antibodies for specificity and sensitivity for carcinoma cells versus reactive mesothelial cells in the diagnosis of metastatic carcinoma in cavity fluids. Currently we are investigating the use of *in situ* hybridization as an ancillary diagnostic technique.

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 β_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 currently 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 extrasosseous 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 extraskelatal 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 recently 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.

Cytogenetic analysis has been proposed as an adjunct diagnostic technique in childhood tumors. A reciprocal 11;22 (q24;q12) translocation has been found in Ewing's sarcoma and PNET. Alveolar rhabdomyosarcoma frequently exhibits a 2;13 translocation, and embryonal rhabdomyosarcoma has shown loss of heterozygosity at loci of chromosome 11. Dr. Tsokos' group has recently identified an 11;22 (q24;q12) translocation in two rhabdomyosarcoma cell lines, one of which shows, in addition, a 2;13 translocation, and in an ectomesenchymoma cell line. These findings suggested that neural features may be more frequent than previously thought in childhood rhabdomyosarcoma, even in those cases without an overt mixed phenotype. This feature may account for a more aggressive behavior in some of these tumors.

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.

Possible histologic predictive parameters were evaluated by Dr. Tsokos in 159 cases of rhabdomyosarcoma in collaboration with St. Jude Children's Research Hospital, and statistical analyses were performed by Dr. Wesley at the NIH.

This study led to the final formulation of the NCI classification scheme, proposed by Drs. Tsokos and Triche, as a histologic scheme with predictive value in rhabdomyosarcoma. The NCI scheme was subsequently evaluated by pathologists from the USA and Europe along with other proposed schemes in 800 rhabdomyosarcoma cases from the Intergroup Rhabdomyosarcoma Study. The data were analyzed statistically by Drs. Geehan and Kashgarian and an international classification scheme has been formulated by the participants (International Classification Committee). This international classification scheme of rhabdomyosarcoma preserves the main subcategories of the previous WHO classification scheme (embryonal, botryoid, alveolar, pleomorphic), but incorporates in the alveolar subcategory the so-called solid alveolar rhabdomyosarcoma, a term proposed by Drs. Tsokos and Triche to define a rhabdomyosarcoma of poor prognosis, but often misclassified as embryonal rhabdomyosarcoma.

Intrinsic differences between embryonal and alveolar rhabdomyosarcoma have also been detected by Dr. Tsokos in rhabdomyosarcoma cell lines studied for c-myc expression, as well as tumorigenicity and metastatic potential in nude mice. This study is currently in press. Briefly, tumorigenicity and metastatic potential were higher in alveolar rhabdomyosarcoma cell lines which showed a uniformly greater over-expression of c-myc gene when compared to the embryonal rhabdomyosarcoma cell lines.

Another method considered to be of prognostic significance in several solid tumors, including Wilms' tumor and neuroblastoma, is slow cytometric analysis of DNA ploidy. Dr. MacLeod, a fellow in the Ultrastructural Pathology Section at the time, modified the Hedley's technique for optimal DNA recovery from paraffin sections, and analyzed the cellular DNA content of approximately 50 rhabdomyosarcoma cases. Flow cytometric and light microscopic evaluation for c-myc staining were also performed. The data are in the process of being analyzed. Possible predictive parameters will be searched for in relation to final clinical outcome.

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 differentiation 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 MyoD₁ RNA and protein expression after 5-aza treatment suggested activation of muscle determination genes as a possible mechanism of action.

Transforming growth factor (TGF- β), 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 MyoD₁ gene expression. Immunohistochemical studies with antibodies against TGF- β ₁ and TGF- β ₃ 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- β 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- β 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- β in rhabdomyosarcoma is currently evaluated in the section using blocking monoclonal antibodies and cDNA probes to detect levels of expression TGF- β -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. 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 motility stimulating cytokine, AMF. 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. 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 imidazole 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 will begin within the next nine months.

Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. The section offers expertise in the diagnosis of hematopoietic disorders for patients admitted to the National Institutes of Health. The staff collaborate closely with physicians treating patients with neoplastic and reactive hematologic and lymphoproliferative disorders. While the emphasis is on clinical protocols based in the NCI, collaborations exist with physicians in NIAID, NHLBI, NEI, and NIAMS. Dr. Jaffe supervises an internationally recognized consultation service which receives over 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 immunohistochemistry and flow cytometry. The flow cytometry laboratory utilizes a FACS scan and a FACS star, and is supervised by Dr. Maryalice Stetler-Stevenson. The section also offers studies in applied molecular diagnosis, using DNA and RNA probes. The diagnostic molecular biology laboratory is supervised by Dr. Mark Raffeld. These facilities are all integrated in the fellowship program in hematopathology.

The Hematopathology Section has published a number of important studies on the clinicopathologic and immunophenotypic aspects of malignant lymphoma. Dr. Jaffe described a unique association of nodular lymphocyte predominant Hodgkin's disease 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 sporadic 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 κ . Three cases which demonstrated a rearrangement involving the switch α 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 which 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 CCCTCCCA. 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 identification 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. 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. Several novel homeobox genes that are expressed in developing limbs have been identified. One of these genes has been further analyzed by *in situ* hybridization and shows a very restricted domain of expression within the limb bud, correlating with the position of a functional zone regulating pattern. Experiments are currently underway to assess the developmental function of this novel homeobox gene using transgenic mice and using avian retroviral expression vectors for transient expression experiments in chick embryos.

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 on five clones have been completed and have revealed two functional classes of proteins encoded by these genes: lymphokines and DNA binding proteins/transcription factors. Potential functional activities of the three putative lymphokines currently are being tested with recombinant proteins.

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

PROJECT NUMBER

Z01 CB 00853-38 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

OTHER: (see next page)

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

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

The Surgical Pathology Section provides service in anatomic pathology for the Clinical Center patients and collaborates with the research staff of all institutes in those investigations which involve the use and study of human pathological material. The frozen section and surgical pathology processing area was constructed adjacent to the new operating rooms and has been in use since April, 1983. This new facility has greatly enhanced processing of specimens and communication of diagnostic findings with attending physicians. It is equipped with intercom and television viewing screens in each operating room to facilitate communication.

The staff is actively engaged in a variety of projects involving clinicopathological correlation and pathologic characterization of diseases studied at the Clinical Center. Up-to-date immunohistochemical techniques have been applied to the study of tumors and other non-neoplastic diseases. The use of immunohistochemical staining has greatly facilitated more precise diagnosis in selected cases and with the increasing number of monoclonal antibodies available, this technique should have even greater value in diagnostic and research pathology. A major renovation of the histology laboratory is in the final stage. This will include space allocated for performance of special stains and immunocytochemistry.

Other Professional Personnel:

G. Jaffe	Expert	LP NCI
C. Axiotis	Expert	LP NCI
+H. Hollingsworth	Medical Staff Fellow	LP NCI
+K. Gardner	Medical Staff Fellow	LP NCI
+R. Doms	Medical Staff Fellow	LP NCI
+J. Taubenberger	Medical Staff Fellow	LP NCI
+D. Kleiner	Medical Staff Fellow	LP NCI
+D. Roth	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
*J. Stern	Consultant in Dermatopathology	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.

Histopathologic and immunohistology studies will be performed as part of the following clinical protocols: 1) Dose intensive chemotherapy in locally advanced and metastatic breast cancer; 2) Use of monoclonal antibody L6 to scintigraphically detect metastases; 3) Combination radioiodine and adriamycin for follicular thyroid cancer; 4) Treatment of stage I and II carcinoma of breast, mastectomy vs. lumpectomy; and 5) Phase II evaluation of suramim in advanced stage carcinoma of prostate.

*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:

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Sandrock D, Merino MJ, Norton J, Neumann RD. Parathyroid imaging by Tc-Tl scintigraphy. *Eur J Nucl Med* 1990;16:607-13.

Kragel PJ, Williams J, Emory T, Merino MJ. Renal oncocytoma with cylindromatous changes: pathologic features and histogenetic significance. *Mod Pathol* 1990;3:277-81.

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Anglard P, Tory K, Brauch H, Weiss GH, Latif F, Merino MJ, Lerman MI, Zbar B, Linehan WM. Molecular analysis of genetic changes in the origin and development of renal cell carcinoma. *Cancer Res* 1991;51:1071-7.

Monteagudo C, Merino MJ, LaPorte N, Neumann RD. Value of gross cystic disease fluid protein-15 in distinguishing metastatic breast carcinomas among poorly differentiated neoplasms involving the ovary. *Hum Pathol* 1991;22:368-72.

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- Jennings TA, Axiotis CA, Kress Y, Carter D. Primary malignant melanoma of the lower respiratory tract: Report of a case and literature review. *Am J Clin Pathol* 1990;94:649-55.
- Nova MP, Kress Y, Jennings TA, Halperin AJ, Axiotis CA. Papillary eccrine adenoma and low grade papillary eccrine carcinoma: A comparative histologic, ultrastructural, and immunohistochemical study. *Surg Pathol* 1990;3:179-87.
- Sugarbaker PH, Landy D, Jaffe G, Pascal R. Histologic changes induced by intraperitoneal chemotherapy with 5-fluorouracil and mitomycin C in patients with peritoneal carcinomatosis from cystadenocarcinoma of the colon or appendix. *Cancer* 65:1990;1495-1501.
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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09145-07 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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, Pulmonary and Postmortem Section, and
Ultrastructural Pathology Section, LP, NCI

LAB/BRANCH

Office of the Clinical Director, NINDS

SECTION

OCD

INSTITUTE AND LOCATION

NINDS, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

A

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

As described previously, subspecialty expertise in diagnostic neuropathology is provided to the Laboratory of Pathology, NCI, and to all other institutes, via the Office of the Clinical Director, 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 case material includes dementia and other degenerative neurological diseases, malignant gliomas, AIDS, 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 35 intra-operative frozen section consultations are provided yearly. Current case material includes malignant gliomas, pituitary adenomas, stereotactic biopsies, and electrocorticographically-guided resections for temporal lobe seizures.

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); white matter degeneration in the elderly (NIA).
2. Pituitary adenomas: study of adenomas, particularly in Cushing's disease; TSH-producing adenomas (NICH, NIDDK, NINDS).
3. Malignant gliomas: diagnosis and grading of tumors prior to experimental therapy; complications of therapy (NINDS, NCI).
4. AIDS (pediatric): study of patients with AIDS encephalopathy.
5. Multiple sclerosis: correlation of acute lesions with neuroimaging.

Publications:

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09192-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

SUMMARY OF WORK (Use standard 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.

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

PROJECT NUMBER

Z01 CB 09193-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

SUMMARY OF WORK (Use standard 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.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09194-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

P-Glycoprotein Expression in Normal Secretory Gestational Endometrium

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
	C. Axiotis	Expert	LP NCI
OTHER:	M. Batista	Senior Staff Fellow	CC
	L. Nieman	Senior Staff Fellow	CC
	R. Neumann	Chief, Nuclear Medicine Department	CC

COOPERATING UNITS (if any)

Endocrinology Branch, NICHD

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

The multidrug-resistance (MDR) gene product, P-glycoprotein (P170) expressed in tumor cells, has also been localized in the apical regions of secretory epithelial cells of the pregnant mouse endometrium. It has also been shown that progesterone interacts with P170 in gravid mouse endometrium. We will study the expression and localization of P170 in the human endometrium of normal controls and patients undergoing therapeutic D&C to investigate if the expression of P170 correlates with progesterone levels of the luteal phase and pregnancy and if P170 plays a role in sustaining progesterone levels necessary to maintain pregnancy.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09359-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	P. Fernandez	Visiting Fellow	LP NCI
	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:

1.5

PROFESSIONAL:

1

OTHER:

.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.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09360-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

The Role of Base Membrane Proteins in Trophoblast Implantation Sites

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

PI:	M. Merino	Chief, Surgical Pathology Section	LP NCI
OTHER:	P. Fernandez	Visiting Fellow	LP NCI
	L. Liotta	Chief, Tumor Invasion & Metastases Section	LP NCI
	W. Stetler- Stevenson	Senior 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:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

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

We will study the synthesis of basement membrane proteins, laminin and type IV collagen as well as the role of enzymes such as collagenase IV in trophoblastic implantation sites.

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

PROJECT NUMBER

Z01 CB 09361-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	C. Axiotis	Expert	LP NCI
OTHER:	K. Cowan	Chief, Medical Breast Cancer Section	MB 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:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

SUMMARY OF WORK (Use standard 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.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09165-04 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Pulmonary and Postmortem Pathology

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

PI: W. Travis Chief, Pulmonary and Postmortem Pathology Section LP NCI

OTHER: C. Baker, P. Howley, D. Levens, L. Liotta, S. Mackem, T. O'Leary, A. Lerner, A. Ginsberg, G. Jaffe, K. Schmidt, J. Taubenberger, D. Roth, D. Kleiner, H. Hollingsworth, K. Gardner, R. Doms, S. Barksdale, C. Moskaluk, T. Giordano, L. Ritchie, A. Dock, J. Rainey, W. Roberts, and D. Katz

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Pulmonary and Postmortem Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

23

PROFESSIONAL:

20

OTHER:

3

CHECK APPROPRIATE BOX(ES)

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

A

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

The Section of Pulmonary and Postmortem Pathology, together with the Cytopathology Section, Hematopathology Section, Surgical Pathology Section, and Ultrastructural Pathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material. The Autopsy Suite has equipment specially designed 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 utilized to protect our staff and residents from exposure to tissues contaminated with high risk infectious agents and radiation.

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to 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. Data from the autopsies from cancer patients treated with interleukin-2 and other forms of immunotherapy have been recently published.

Objectives:

The objectives of the Pulmonary and Postmortem Section are: (a) to provide diagnostic services in pulmonary and autopsy pathology for the clinical research projects conducted at NIH. This includes generating pathology reports for current cases and presenting the pathologic findings of autopsies and lung biopsies at clinical conferences with clinicians and radiologists; (b) to carry out independent research; (c) to provide teaching to the NIH residency program in anatomic pathology; and (d) to collaborate with investigators in research involving the use and study of human materials.

The proposed research program includes (a) continuing to provide the services described; (b) increasing interaction with clinical branches in the design and evaluation of protocols; (c) providing opportunities for residents to participate in teaching, and in research projects; (d) developing data retrieval systems for the autopsy and pulmonary pathology material.

Three projects are currently in progress on the pulmonary pathology of AIDS.

- 1) We have reviewed all of the lung specimens from autopsies on AIDS patients through 1988. These data have been written up as a clinical paper focusing on the contribution of pulmonary pathology to the cause of death in AIDS patients. We plan to do an updated review of all AIDS autopsies through 1991 with emphasis on the pathology.
- 2) The lung biopsy and autopsy specimens from patients with pulmonary Kaposi's sarcoma have been reviewed and are being written up into a manuscript.
- 3) The lung biopsy specimens from patients with lymphocytic interstitial infiltrates have been written up. These cases include examples of lymphocytic interstitial pneumonitis and nonspecific interstitial pneumonitis.

Publications:

Atkinson JC, Travis WD, Pillemer SR, Bermudez D, Wolff A, Fox PC. Major gland salivary function in primary Sjögren's syndrome and its relationship to clinical features. *J Rheumatol* 1990;17:318-22.

Devaney KO, Travis WD, Hoffman G, Leavitt R, Lebovics R, Fauci AS. Interpretation of head and neck biopsies in Wegener's granulomatosis. *Am J Surg Pathol* 1990;14:555-64.

Kragel A, Pittaluga S, Travis WD, Feinberg L, Lotze M, Yang J, Rosenberg SA. Pathologic findings associated with immunotherapy for cancer: a postmortem study of 19 patients. *Hum Pathol* 1990;21:493-502.

Travis WD, Lack EE, Azumi N, Tsokos M, Norton J. Adenomatoid tumor of the adrenal gland with ultrastructural and immunohistochemical evidence of a mesothelial origin. *Arch Pathol Lab Med* 1990;114:722-4.

Kragel PJ, Pestaner J, Travis WD, Linehan WM, Filling-Katz MR. Papillary cystadenoma of the epididymis: a report of three cases with lectin histochemistry. Arch Pathol Lab Med 1990;114:672-5.

Hauser GJ, McIntosh JK, Travis WD, Rosenberg SA. Manipulation of oxygen radical-scavenging capacity in mice alters host sensitivity to tumor necrosis factor toxicity but does not interfere with its antitumor efficacy. Cancer Res 1990;50:3503-8.

Pass HI, Doppman JL, Nieman L, Stovroff M, Vetto J, Norton JA, Travis WD, Chrousos GP, Oldfield EH, Cutler GB. Management of patients with thoracic ectopic ACTH syndrome. Ann Thorac Surg 1990;50:52-7.

Puri RK, Travis WD, Rosenberg SA. Recombinant interferon- α and recombinant interleukin-2 induced proliferation of lymphoid cells *in vivo* in the organs of mice. Cancer Res 1990;50:5543-50.

Kragel AH, Travis WD, Steis RG, Rosenberg SA, Roberts WC. Myocarditis or acute myocardial infarction associated with interleukin-2 therapy for cancer. Cancer 1990;66:1513-6.

Kragel PJ, Devaney KO, Meth BM, Frierson HF, Linnoila RI, Travis WD. Mucinous cystadenoma of the lung: a report of two cases with immunohistochemical and ultrastructural analysis. Arch Pathol Lab Med 1990;114:1053-6.

Perry RR, Keiser HJ, Norton JA, Wall RT, Robertson CN, Travis WD, Pass HI, Walther MW, Linehan WM. Surgical management of pheochromocytoma with the use of metyrosine. Ann Surg 1990;212:621-8.

Ehrenreich H, Anderson RW, Fox CH, Rieckmann P, Hoffman GS, Travis WD, Coligan JE, Kehrl JH, Fauci AS. Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. J Exp Med 1990;172:1741-8.

Valdez IH, Katz RW, Travis WD. Premature alveolar bone loss in Erdheim-Chester disease. Oral Surg, Oral Med, Oral Pathol 1990;70:294-6.

Katz RW, Brahim JS, Travis WD. Oral squamous cell carcinoma arising in a patient with long-standing lichen planus. A case report. Oral Surg, Oral Med, Oral Pathol 1990;70:282-5.

Patton LL, Brahim JS, Travis WD. Mandibular osteomyelitis in a patient with sickle cell anemia: report of case. J Am Dent Assoc 1990;121:602-4.

Lack EE, Schloo BL, Azumi N, Travis WD, Grier H, Kozekewich HPW. Undifferentiated (embryonal) sarcoma of the liver: a study of 16 cases with clinicopathologic correlation and emphasis on immunohistochemical features. Am J Surg Pathol 1991;15:23-31.

Doppman JL, Pass HI, Nieman L, Findling JW, Dwyer AJ, Feuerstein IM, Ling A, Travis WD, Cutler GB, Chrousos GP, Loriaux DL. Magnetic resonance imaging for detection of ACTH-producing bronchial carcinoid tumors. Am J Roentgenol 1991;156:39-44.

Fraker DL, Travis WD, Merendino JJ, Zimring MB, Streeten EA, Weinstein LS, Marx SJ, Spiegel AM, Aurbach GD, Doppman JL, Norton JA. Locally recurrent parathyroid neoplasms as a cause for recurrent and persistent primary hyperparathyroidism. Ann Surg 1991;213:58-65.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09166-04 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 and Postmortem 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 and Postmortem Pathology 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.)

A detailed pathologic review of the NIH (Pulmonary Branch, NHLBI) experience with pulmonary interstitial fibrosis is being performed to investigate potential new approaches to the diagnosis and pathologic subclassification of interstitial fibrosis. Recent reports have described newly recognized forms of interstitial fibrotic lung disease previously classified as idiopathic pulmonary fibrosis suggesting a need for rethinking of traditional concepts of the pathology of pulmonary interstitial fibrosis.

The broad experience of the Pulmonary Branch, NHLBI, provides a rich resource of clinical and pathologic material which may provide the basis for recognition of new prognostically significant forms of interstitial lung fibrosis.

Lung biopsies from 60 patients with idiopathic pulmonary fibrosis have already been reviewed and the data are currently being analyzed. Biopsies from 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.

Publications:

Travis WD, Pittaluga S, Lipschik GY, Ognibene FP, Suffredini AF, Lane HC, Kovacs J, Masur H, Pass HI, Shelhamer JH. Atypical pathologic manifestations of *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome: Review of 123 lung biopsies from 76 patients with emphasis on cavities, vascular invasion, vasculitis and granulomas. *Am J Surg Pathol* 1990;14:615-25.

Travis WD, Colby TV, Lombard C, Carpenter HA. A clinicopathologic study of 34 cases of diffuse pulmonary hemorrhage with lung biopsy confirmation. *Am J Surg Pathol* 1990;14:1112-25.

Hoffman GS, Schler JMG, Gallin JI, Shelhamer JH, Suffredini A, Ognibene FP, Baltaro R, Fleisher TA, Leavitt RY, Travis WD, Barile MF, Tsokos M, Strauss SE, Holman RP, Fauci AS. The use of bronchoscopy and bronchoalveolar lavage to study the pathophysiology of Wegener's granulomatosis. *Am Rev Respir Dis* 1991;143:401-7.

Rom WN, Travis WD, Brody AN. Cellular and molecular basis of the asbestos-related diseases. *Am Rev Respir Dis* 1991;143:408-22.

Travis WD, Leavitt R, Hoffman G, Pass HI, Fauci A. The spectrum of pulmonary pathologic findings in Wegener's granulomatosis; review of 87 biopsies from 67 patients. *Am J Surg Pathol* 1991;15:315-33.

Travis WD, Roth DB. Histopathologic evaluation of lung biopsy specimens. In: Shelhamer J, Pizzo PA, Parrillo JE, Masur H, eds. *Respiratory disease in the immunosuppressed host*. Philadelphia: JB Lippincott Co, 1991;182-217.

Leavitt RY, Travis WD, Fauci AS. Vasculitis. In: Shelhamer J, Pizzo PA, Parrillo JE, Masur H, eds. *Respiratory disease in the immunosuppressed host*. Philadelphia: JB Lippincott Co, 1991;703-27.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00852-38 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	L. Elwood	Medical Officer	LP NCI
	C. Copeland	Cytotechnologist	LP NCI
	L. Galito	Biologist	LP NCI
	A. Wilder	Cytotechnologist	LP NCI
	E. Sanders	Bio. Lab. Technologist	LP NCI
	C. King	Medical Technologist	LP NCI
	T. Howard	Secretary	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

7

PROFESSIONAL:

2.5

OTHER:

4.5

CHECK APPROPRIATE BOX(ES)

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

A

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

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 454A12MAB-RICIN A chain conjugate given intrathecally for refractory carcinomatous meningitis. Our collaborative effort in this project involves the cytomorphologic evaluation of cerebrospinal fluid (CSF) specimens in order to: 1) document the presence of malignancy in the CSF prior to initiation of intrathecal immunotoxin therapy; 2) establish the baseline CSF tumor burden prior to therapy; and 3) monitor the cytologic response quantitatively throughout the post-therapy period.

Another collaborative project with critical care medicine is comparing the diagnostic sensitivities of bronchoalveolar lavage, sputum, transbronchial biopsy, and various culture and immunocytochemical techniques in the detection of CMV in HIV positive patients.

Major Findings:

Approximately 3600 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 200% over previous years.

Cytological techniques are utilized in collaborative work with other sections and branches of NIH. For example, single cell tumor suspensions, tumor cell lines, and stimulated lymphocyte cultures are evaluated microscopically and by immunocytochemical techniques.

Publications:

Solomon D, Jaffe G. Cytopathologic diagnosis of respiratory diseases in the immunosuppressed host. In: Shelhamer J, Pizzo PA, Parrillo JE, Masur H, eds. Respiratory diseases in the immunosuppressed host. Philadelphia: JB Lippincott, 1991;218-31.

Elwood L, King C, Colandrea J. Urinary cytology. In: Atkinson BF, ed. Atlas of diagnostic cytopathology. WB Saunders (in press)

Elwood L, Dobrzanski D, Feuerstein I, Solomon D. *Pneumocystis carinii* in pleural fluid: The cytologic appearance. Acta Cytol (in press)

Manyak MJ, Nelson LM, Solomon D, DeGraff W, Stillman RJ, Russo A. Fluorescent detection of rabbit endometrial implants resulting from monodispersed viable cell suspensions. Fertil Steril 1990;54:356-9.

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

PROJECT NUMBER

Z01 CB 00897-08 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	L. Elwood	Medical Officer	LP NCI
	C. King	Medical Technologist	LP NCI
	J. Colandrea	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:

1.0

PROFESSIONAL:

.5

OTHER:

.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 lymphoma.

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.

Major Findings:Immunocytochemistry in the evaluation of lymphoid cell populations:

We have investigated 460 specimens, including 229 pleural and peritoneal effusions, 102 cerebrospinal fluids, and 142 fine needle aspiration specimens. We have found 219 cases to be positive for lymphoma and 137 to be reactive in nature. Of the 219 positive cases, 178 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 36 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:

Pluda JM, Yarchoan R, Jaffe ES, Feuerstein IM, Solomon D, Steinberg SM, Marczyk KS, Raubitschek A, Katz D, Broder S. Development of opportunistic non-Hodgkin's lymphoma in a cohort of patients with severe HIV infection on long-term antiretroviral therapy. *Ann Intern Med* 1990;113:276-82.

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

PROJECT NUMBER

Z01 CB 09153-05 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	C. King	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

Surgery Branch, DCT

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

.20

OTHER:

.20

CHECK APPROPRIATE BOX(ES)

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

D

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

Clinical trials employing the adoptive transfer of expanded tumor infiltrating lymphocytes to patients with metastatic disease are currently underway under the direction of the Surgery Branch, NCI. Our collaborative effort in this project involves immunocytochemical analysis of tumor cell suspensions to identify (1) the percentage and phenotypic expression of subsets of tumor infiltrating lymphocytes present in the tumor and (2), tumor markers, if any, which are expressed by the tumor cells. Once the tumor infiltrating lymphocyte cultures have been expanded and are to be harvested for patient therapy, we analyze the material using routine cytologic preparations and immunocytochemistry to ensure the cultures are free of tumor cells.

Major Findings:

Over 220 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 350 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:

Haas GP, Solomon D, Rosenberg SA. Tumor infiltrating lymphocytes from non-renal urologic malignancies. Cancer Immunol Immunother 1990;30:342-50.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09176-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Quality Assurance in Cervical/Vaginal Cytopathology

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

PI:	D. Solomon	Chief, Cytopathology Section	LP NCI
OTHER:	C. King	Medical Technologist	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:

.40

PROFESSIONAL:

.25

OTHER:

.15

CHECK APPROPRIATE BOX(ES)

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

A

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

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 at the National Institutes of Health in Bethesda, Maryland with a capacity attendance. The two day meeting provided open exchange of data, lively debate and a forum for critical analysis of TBS. Modifications proposed at the April conference will be reviewed by an Editorial Committee composed of pathologists, cytologists, and gynecologists which will draft *The Bethesda System II*. The Cytopathology Section has functioned as the principal organizer of the meeting. I will continue to be involved in the Editorial Committee and the dissemination of TBS through presentations at national and international meetings.

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; the Laboratory Initiatives for the Year 2000 Health Objectives for the Nation; an NCI Working Group to assess the current role of cytopathology in cervical cancer screening; 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. Quality assurance in cytopathology: The role of the government. *Analyt Quantit Cytol Histol* 1990;12:212.

Solomon D. Does the Bethesda system promote or endanger the quality of cervical cytology? [Letter to the Editor]. *Acta Cytol* 1990;34:456.

Solomon D. Letter to the Editor. *J Reprod Med* 1990;35:541.

Solomon D. The 1988 Bethesda system. [Letter to the Editor]. *Acta Cytol* 1990;34:903.

Kline TS, Solomon D. Guidelines for specimen adequacy: A plea for workable definitions. *Diag Cytopathol* 1991;7:1-2.

Kurman R, Malkasian G Jr, Sedlis A, Solomon D. Clinical commentary: From Papanicolaou to Bethesda. *Obstet Gynecol* 1991;77:779-82.

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

PROJECT NUMBER

Z01 CB 09178-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Immunophenotypes of T Cells and Stromal Cells in Mouse Peyer's Patches

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

PI:	L. Elwood	Medical Officer	LP NCI
OTHER:	G. Harriman	Medical Staff Fellow	LCI NIAID
	C. King	Medical Technologist	LP NCI

COOPERATING UNITS (if any)

Mucosal Immunity Section, NIAID

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.20

PROFESSIONAL:

0.10

OTHER:

0.10

CHECK APPROPRIATE BOX(ES)

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

A

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

The mechanisms involved in B-cell activation and isotype switching in the germinal centers of Peyer's patches are poorly understood. In an attempt to better define some of the factors involved, studies in mice have been underway to characterize the B-cell-associated T-cells in mouse Peyer's patches by flow cytometry and to isolate B-cell associated stromal cells. In our laboratory, we have used frozen section immunohistochemistry to further characterize the various T-cell subsets found by flow cytometry. The role of other factors such as transforming growth factor- β 1 is currently being assessed.

Publications:

Harriman GR, Lycke NY, Elwood LJ, Strober W. T lymphocytes that express CD4 and the alpha beta-T cell receptor but lack Thy-1. Preferential localization in Peyer's patches. J Immunol 1990;145:2406-14.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09186-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Bone Marrow Effects of Interleukin-1 Alpha

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

PI:	L. Elwood	Medical Officer	LP NCI
OTHER:	J. Smith	Senior Investigator	BRMP CRB DCT NCI
	R. Steis	Senior Investigator	BRMP CRB DCT NCI
	D. Longo	Associate Director	BRMP CRB DCT NCI

COOPERATING UNITS (if any)

BRMP, DCT, NCI

LAB/BRANCH

Laboratory of Pathology

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Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.25

PROFESSIONAL:

.10

OTHER:

.15

CHECK APPROPRIATE BOX(ES)

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

A

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

Interleukin-1 (IL-1) is a polypeptide produced by human cells that has a wide range of biological effects and is involved in responses to inflammatory, immunologic and infectious challenges. The genes for two different forms of IL-1, IL-1 alpha and IL-1 beta, have been cloned allowing *in vitro* production of large quantities of these cytokines.

IL-1 alpha has been shown to have immunoenhancing, bone marrow restorative and direct anti-tumor effects. Because of these effects, it was postulated that IL-1 alpha could have significant clinical utility in the treatment of malignant disease and a phase I study of its toxicity and hematologic and immunologic effects in humans was undertaken. My role has been to assess pre- and post-treatment bone marrow aspirates and peripheral blood smears in patients with metastatic malignant disease given increasing doses of IL-1 alpha.

Major Findings:

To date, pre- and post-treatment marrows and peripheral blood smears have been evaluated in 25 patients treated with IL-1 alpha. Treatment resulted in a marked rise in the total WBC from 2 to 7 times above baseline. This rise was dose related, decreasing with time during treatment, and was due predominantly to an increase in mature neutrophils.

Post-treatment bone marrow aspirates showed marked increases in cellularity relative to pre-treatment marrows in patients receiving a threshold dose of IL-1 alpha. The increased cellularity was due to an increase in granulocyte precursors with proportionate increases at all levels of maturation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09350-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Immunocytochemistry of Glutathione S-transferase-pi in Breast and Uterine Cervix

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:	L. Elwood	Medical Officer	LP NCI
	L. Gilbert	Fogarty Fellow	COP DCT NCI

COOPERATING UNITS (if any)

DCT LP

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

.25

PROFESSIONAL:

.25

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

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

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.

Preliminary studies utilizing immunohistochemistry have also demonstrated increased GST-pi expression in preneoplastic and neoplastic lesions of the uterine cervix. We are further evaluating this relationship using immunocytochemistry in cervical/vaginal biopsies, as well as cervical/vaginal smears. In addition, *in situ* hybridization for HPV DNA performed on the same specimens will enable us to explore possible relationships between GST-pi overexpression and various HPV types.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09351-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

The Revision of Proposed Federal Cytology Regulations Implementing CLIA '88

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

PI:	L. Elwood	Medical Officer	LP NCI
OTHER:	B. Addison	Assistant Director for Regulatory Affairs	PHPPD DLS CDC
	E. Plott	Cytotechnologist	CPCB CCDPHP CDC
	D. Howerton	Health Scientist	PHPPD LPB CDC

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TOTAL MAN-YEARS:

.15

PROFESSIONAL:

.15

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A

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

On October 31, 1988, Congress enacted the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) which included specific standards for laboratories providing cytology services. On May 21, 1990, the Department published proposed regulations for implementing CLIA '88 with an open comment period of 90 days. These proposed regulations were developed by the Health Care Financing Administration (HCFA) with technical support from the Center for Disease Control (CDC). The CDC staff included health scientists and cytotechnologists.

The comments received during the comment period were overwhelming in number and degree of concern with the regulations. A committee has been assembled to evaluate the comments and, accordingly, to emend the proposed regulations. I am providing the sole cytopathologist expertise to this panel.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09187-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Transforming Growth Factor (TGF)- β in the Differentiation of Neuroblastoma & PNET

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

PI:	M. Tsokos	Chief, Ultrastructural Pathology	LP NCI
OTHER:	B. McCune	Biotechnology Fellow	LC NCI
	M. Sporn	Laboratory Chief	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:

1

PROFESSIONAL:

3/4

OTHER:

1/4

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- β 1, 2, and 3 by neuroblastoma cells, except for well differentiated ganglion cells in ganglioneuroblastomas and in normal ganglia. In contrast, almost all primitive neuroectodermal tumors (PNET) expressed variable amounts of TGF- β 1 and 3. Absence of TGF- β secretion was also found in conditioned media from neuroblastoma cell lines, in contrast to PNET cell lines using a bioassay based on TGF- β inhibition of DNA synthesis in mink lung fibroblasts. These data suggested a possible role of TGF- β in the differentiation of human neuroblastoma. This hypothesis will be further pursued in the following ways: (1) TGF- β expression and secretion will be studied in neuroblastoma and PNET cell lines before and after differentiation with known differentiating agents, such as retinoic acid and TPA. (2) Direct effects of exogenously added TGF- β in undifferentiated and differentiated neuroblastoma cell cultures will be determined. (3) Blocking antibodies and antisense TGF- β oligonucleotides will be used to modulate possible actions of TGF- β on neuroblastoma and PNET cells *in vitro*. Preliminary data have shown induction of TGF- β 1-mRNA in SH-SY5Y neuroblastoma cells following treatment of cultures with TPA or retinoic acid, both of which promoted differentiation. In contrast, IMR-32 neuroblastoma cells which showed minimal morphologic differentiation with the same agents showed only mild increase in TGF- β 1 mRNA levels.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09354-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

The Role of Transforming Growth Factor (TGF)- β in Rhabdomyosarcoma

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

PI:	M. Tsokos	Chief, Ultrastructural Pathology	LP NCI
OTHER:	J. Keleti	Visiting Fogarty Fellow	LP NCI
	B. McCune	Biotechnology Fellow	LC NCI
	M. Sporn	Laboratory Chief	LC NCI

COOPERATING UNITS (if any)

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Laboratory of Pathology

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Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

2/4

OTHER:

2/4

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- β 1, and to a lesser extent TGF- β 3, but lack TGF- β 2 protein by immunohistochemical staining. We also detected variable levels of TGF- β protein synthesis in conditioned media of cultured rhabdomyosarcoma (RMS) cells using a bioassay based on TGF- β inhibition of DNA synthesis in mink lung fibroblasts (CCL-64 cells). Furthermore, all RMS expressed variable levels of TGF- β 1 mRNA *in vitro*. These data, in combination with our previous data of TGF- β -induced inhibition of RMS cell differentiation *in vitro*, and the known inhibitory effect of TGF- β in normal myogenesis, have suggested to us a possible autocrine inhibitory role of TGF- β in human RMS. This hypothesis will be studied in the following ways: (1) RMS cell lines expressing high and low levels of TGF- β mRNA will be studied for the presence of TGF- β receptors by PAGE of cell lysates incubated with 125 I-TGF- β 1,2,3. (2) Anti-TGF- β blocking antibodies and antisense oligonucleotides will be employed to investigate interference with cell growth (DNA synthesis by 3H-thymidine incorporation) and inhibition of myogenic differentiation (myotube fusion assay), in the presence or absence of increased concentration of exogenous TGF- β . Since TGF- β receptors decrease after fusion of myoblasts into myotubes, and our previous data have shown a more potent action of TGF- β in cell lines with a more primitive morphology, we will also evaluate levels of TGF- β mRNA and protein in fusion arrested (with diazepam or EGTA), versus 5-azacytidine differentiated RMS cells. Retinoic acid (RA)-treated cells will be evaluated as well, since RA was found to induce TGF- β receptor expression in some cell types.

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

PROJECT NUMBER

Z01 CB 09355-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Immunohistochemical Detection of Wild and 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 LP NCI
OTHER: Y. Hijazi Senior Staff Fellow LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

2/4

OTHER:

2/4

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. 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. Recently, antibodies specific to wild or mutant p53 proteins have become commercially available. We will use these antibodies to stain rhabdomyosarcomas of the alveolar and embryonal histologic subtype, before and after disease progression, known clinical parameters, such as clinical stage and outcome. This study will help us answer questions as to a possible role of p53 gene product in human rhabdomyosarcoma, its possible relationship to a specific (? more aggressive) histologic subtype, and its potential role in the identification of rhabdomyosarcomas with more aggressive clinical behavior.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09172-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Biotechnology Fellow	LP NCI
	N. Guo	Visiting Fellow	LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.8

PROFESSIONAL:

1.8

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

The 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 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 (Cancer Res. 48:6875, 1988). 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. Small cell lung carcinoma cells which attach on thrombospondin but not on other adhesive proteins will be used to identify specific thrombospondin receptors. The intracellular responses of cells to binding of thrombospondin on the two types of receptors are also being investigated. Expression of thrombospondin mRNA in tumor cells is being examined to look for association of thrombospondin synthesis with *in vivo* metastatic potential.

Major Findings:

1. Suramin is a polysulfonated drug that inhibits binding of some growth factors to cells, inhibits of tumor cell growth, and glycosaminoglycan metabolism. Suramin also inhibits binding of the adhesive glycoproteins thrombospondin and laminin to immobilized sulfate with ID50 values of 220 $\mu\text{g/ml}$ and 470 $\mu\text{g/ml}$, respectively. Suramin at 50 to 400 $\mu\text{g/ml}$ specifically inhibited G361 melanoma cell spreading on thrombospondin without affecting cell attachment, inhibited spreading of A2058 melanoma cells on thrombospondin and laminin, and partially inhibited A2058 cell attachment. However, suramin had no effect on G361 or A2058 cell attachment or spreading on fibronectin. Chemotaxis of melanoma cells to thrombospondin and laminin were also specifically inhibited by suramin, as was haptotaxis to laminin. However, suramin only weakly inhibited haptotaxis of melanoma cells to thrombospondin, which is not mediated by the amino-terminal domain, and did not inhibit haptotaxis to fibronectin. 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.
2. Small cell lung carcinoma cells adhere specifically on thrombospondin but not on other adhesive matrix components including laminin, fibronectin, type I or IV collagen, or vitronectin. Thrombospondin binding is mediated by the 140 kDa carboxyl terminal domain and is not dependent on sulfated glycoconjugates. Equilibrium binding demonstrated 1.4×10^5 sites with a dissociation constant of 51 nM. The thrombospondin receptor is calcium-dependent and trypsin-sensitive.
3. Cellular responses to binding of thrombospondin to the two classes of receptors are being investigated. Changes in intracellular inositol phosphate and cyclic AMP were observed following exposure of A2058 melanoma cell monolayers to thrombospondin. Thrombospondin at 5 $\mu\text{g/ml}$ causes a concentration- and time-dependent inhibition in inositol trisphosphate and inositol monophosphate levels that is maximal at 2 minutes of incubation. There is a parallel increase in cAMP and a decrease in cGMP to 130% and 60% of controls, respectively. Theophylline, a phosphodiesterase inhibitor, causes a similar decrease in IP_3 and an increase in cAMP. A 140 kDa fragment of thrombospondin generates the same changes in IP_3 and cAMP as thrombospondin, suggesting that the heparin-binding domain is not required for second messenger effects in adherent melanoma cells. Thrombospondin contains an Arg-Gly-Asp sequence near the COOH-terminus of each subunit. Addition of Arg-Gly-Asp peptides causes a rounding of the cell bodies but did not change IP_3 levels. These data suggest that the integrin receptor-binding domain of thrombospondin is not required for generation of the observed second messenger changes, and that these changes are not an indirect result of disruption by thrombospondin of integrin-mediated focal adhesion.

Publications:

Zabrenetzky V, Kohn EC, Roberts DD. Suramin inhibits laminin- and thrombospondin-mediated melanoma cell adhesion and migration and binding of these adhesive proteins to sulfatide. Cancer Res 1990;50:5937-42.

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

PROJECT NUMBER

Z01 CB 09173-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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. Hackstadt, Dept. of Pathology, University of Texas Medical Branch,
Galveston; T. Walsh, Pediatric Oncology, NCI, NIH; R. Mecham, Washington
University Medical Center, St. Louis

LAB/BRANCH

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Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.9

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

Adhesive specificities of *Staphylococcus aureus*, some *Enterococcus* species, *Candida albicans*, and elementary bodies of *Chlamydia trachomatis* are being examined. These have been screened for binding to 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 by 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*.

Chlamydia trachomatis: Several serotypes of Chlamydia were examined for binding to glycolipids and glycoproteins. Three binding specificities were identified from these studies. The elementary bodies bind specifically to glycolipids containing GalNAc β 1-4Gal β 1-4Glc sequences, heparin or fucoidin conjugated to albumin, and to the adhesive proteins fibronectin and laminin. Inhibitors of each of these specificities will be used to determine the role of each in infection of cultured cell monolayers.

Publications:

Roberts DD. Interactions of respiratory pathogens with host cell surface and extracellular matrix components. *Am J Resp Cell Molec Biol* 1990;3:181-6.

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

PROJECT NUMBER

Z01 CB 09174-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	V. Zabrenetzky	Biotechnology Fellow	LP NCI
	N. Guo	Visiting Fellow	LP NCI
	T. Vogel	General Fellow	LP NCI

COOPERATING UNITS (if any)

H. Gralnick, Hematology Service, CC, NIH;
J. Cashel, Biochemical Pathology Section, LP, NCI

LAB/BRANCH

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.9

PROFESSIONAL:

1.4

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

B

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

We have found that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion (Cancer Res. 48:3367, 1988) and as receptors for thrombospondin on melanoma cells (Cancer Res. 48:6785, 1988). Relatively few of the glycolipids belonging to this class have been characterized. We are purifying novel sulfated glycolipids from several sources including human kidney and meconium and breast and small cell lung carcinoma cell lines. The structures of these glycolipids will be examined using chemical and immunological approaches. Monoclonal antibodies to these will be used to examine the potential of these structures as tumor markers. 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:

A sulfatide-binding site on the globular end region of the long arm of laminin has been identified. Following proteolytic digestion with thermolysin, an intact fragment of the laminin A chain carboxyl-terminal domain exhibiting sulfatide-binding activity was isolated using gel filtration and heparin-affinity chromatography. 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, since reduction and alkylation of the fragments abolished sulfatide binding, under conditions in which heparin binding was retained. Thus, sulfatide-binding requires an intact three-dimensional structure. The iodinated fragment bound to A2058 melanoma and T47D breast carcinoma cells and could be displaced by unlabeled fragment. Based on incorporation of [³⁵S]-sulfate, 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 significantly inhibited interaction of labeled intact laminin with melanoma and breast carcinoma cells in direct binding assays.

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 restored 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%).

Publications:

Taraboletti G, Rao CN, Krutzsch HC, Liotta LA, Roberts DD. Sulfatide-binding domain of the laminin A chain. *J Biol Chem* 1990;265:12253-8.

Data RE, Williams SB, Roberts DD, Galnick HR. Platelets adhere to sulfatides by von Willebrand factor dependent and independent mechanisms. *Thromb Haem* (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09175-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Glycolipid 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, Univ. of Texas Med. Branch, Galveston

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

B

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

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 a monoclonal antibody to human secretory component which recognizes glycoproteins and glycolipids produced in colon adenocarcinomas.

Major Findings:

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. 133:2116-2125, 1984), LS-180 and SW1116 cells. Binding of the antibody is lost after treatment of free secretory component with peptide N-glycosidase F (Bakos et al., J. Immunol. 146:162-168, 1991) or periodate, suggesting that 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 sequences bind with much higher affinity. The antibody also binds to type I oligosaccharide sequences substituted with Fucal-4GlcNAc but not with Fucal-2Gal. Milk oligosaccharides containing the Lewis Fucal-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. The antibody binds to some purified lacto-series glycolipids including III⁴αFuc-lactotetraosyl ceramide, lactotetraosyl ceramide, and to neutral glycolipids in human meconium and SW1116 and HT29 adenocarcinoma cell lines. By Western blotting, the antibody 6C4 binds to several proteins in colon adenocarcinoma cell extracts and binds to some purified human mucins.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00891-08 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	E. Schiffmann	Research Chemist	LP NCI
OTHER:	A. Arestad	Special Volunteer	LP NCI
	M. Souroush	Summer Res. Training Program	LP NCI
	H. Krutzsch	Expert	LP NCI

COOPERATING UNITS (if any)

R. Hopkins and P. Harley, PRI/Dyne Corp., FCRDC
T. Vogel, Biotechnology General, Rehovot, Israel

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.9

PROFESSIONAL:

1.6

OTHER:

1.3

CHECK APPROPRIATE BOX(ES)

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

B

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

We have been studying tumor cell motility as a component of the process of metastatic dissemination. The autocrine motility factor (AMF) is synthesized by human melanoma cells and is known to stimulate both directed and random motility in these same cells. AMF has been purified to homogeneity and appears to have a precursor (130 kDa) as well as the previously described 60-65 kDa active component (picomolar activity). The N-terminal sequence of the 60-65 kDa protein has been confirmed with 3 different protein preparations. Oligonucleotides have been synthesized which correspond to the N-terminal amino acid sequence and have been used to screen cDNA libraries made from the same melanoma cell line. In parallel studies, we have looked at the effect of pharmacologic agents which react with components of the cytoskeleton. These agents all inhibit AMF-stimulated motility but have variable effects on cellular adhesion. In a collaborative study, we have looked at a 33 kDa fragment of fibronectin and found this fragment to have the capacity to stimulate full chemotactic and haptotactic responses compared to whole fibronectin.

Major Findings:

We have been studying the role of cell motility in the metastatic dissemination of malignant tumor cells. Our work has focused on agents and mechanisms by which this migration is initiated and controlled. We have identified an autocrine motility factor (AMF) which is secreted by A2058 human melanoma tumor cells and which stimulates these same cells to migrate.

I. AMF Studies

A. Purification of the autocrine motility factor (AMF)

The factor, a protein, has been purified to homogeneity previously but always in quantities too small to obtain N-terminal sequence information. Our efforts during the past year have focused on improving yield in the process of purification. Approximately 200 L of A2058 conditioned medium was prepared by the Frederick Cancer Research Facility and partially concentrated using ultrafiltration membranes that have very low protein binding capacity. We further concentrated this material, then purified the protein with the following sequence of steps. The concentrated conditioned medium was passed over a gelatin-agarose column to remove type IV collagenase and TIMP-2. The AMF-containing material, which did not bind to gelatin, was subjected to 1.2 M ammonium sulfate at 5°C. After centrifugation, this supernatant was chromatographed on a phenyl sepharose column (hydrophobic interaction). Chemotactically active fractions were concentrated and applied to a phenyl TSK column which has finer separation than the phenyl sepharose. These active fractions were washed and separated sequentially by weak anion exchange chromatography, concanavalin A lectin affinity chromatography, and strong anion exchange chromatography. Active material, which had either a 130 kDa band alone or the 130-150 kDa band mixed with a 62 kDa band was subjected to SDS gel electrophoresis and electroblotted to an Immobilon™ matrix. The protein bands could be visualized by Coomassie stain.

The material from the electroblot was subjected to micro-Edman degradation and the previously obtained N-terminal sequence was confirmed for the 62 kDa protein. The 130 kDa fragment appears to be N-terminally blocked and could not be sequenced using this technique.

The N-terminal amino acid sequence of the 62 kDa protein was synthesized in large quantities, cross-linked to bovine serum albumin, and used to immunize rabbits. Serum was purified by ammonium sulfate precipitation followed by peptide affinity chromatography. These purified antibodies were able to neutralize the motility stimulated by partially purified AMF which contained both the 62 and 130 kDa proteins. Western blotting with the antibody revealed a band at 150-180 kDa, as well as weak bands around 65 and 45 kDa.

B. Plans for the AMF study

It is planned to continue with the rabbit antibody studies. Several additional rabbits are being immunized with the N-terminal peptide. In addition, we plan to immunize mice with preparations containing the whole AMF molecules. We will

screen these antibodies using assays to determine direct neutralization of activity, immunoprecipitation of activity, and Western blotting of appropriate bands.

We also plan to continue using oligonucleotides derived from the N-terminal amino acid sequence of the 62 kDa protein to clone AMF by probing cDNA libraries derived from A2058 cells. In addition, the oligonucleotides can serve as templates for PCR amplification of portions of the AMF gene.

Now that these proteins can be isolated and purified, we will begin to search for 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.

II. Cytoskeleton Studies

We have utilized pharmacologic agents which react with the cellular cytoskeleton to determine the effect of these agents on AMF-stimulated motility and on adherence to a variety of substrates. Specifically, we have utilized cis-tubulazole, which inhibits polymerization of microtubules, taxol, which stabilizes microtubules and retards depolymerization, and cytochalasins B and D which inhibit formation of microfilaments. Some of these agents or close relatives have been utilized as antineoplastic drugs, and we hoped to shed insight on their mechanism of action in tumor cells.

All of these anti-cytoskeleton agents inhibited AMF-stimulated motility. The microtubule-specific agents also inhibited adherence to laminin, gelatin, and tissue culture plastic. Adherence to laminin, which has a high affinity receptor on the surface of A2058 cells, was less affected by either microtubule-specific agent than binding to gelatin and tissue culture plastic, both of which appeared to be non-specific or low-affinity ligands. The cytochalasins appeared to have negligible effect on adherence to any of the tested substrates.

III. Studies with a 33 kDa Fibronectin Fragment

We have demonstrated that a 33 kDa fragment of fibronectin, which contains the RGD site, was capable of stimulating both chemotactic and haptotactic migration. This fragment was recombinant, pure by gel electrophoresis, and insensitive to reduction. The concentration of the 33 kDa fragment which was required to elicit a full haptotactic response is approximately 5-fold higher than the necessary concentration of fibronectin (100 nM vs 20 nM, respectively). Approximately 10-fold more of the 33 kDa molecule was required for a maximal chemotactic response compared to fibronectin (300 nM vs 30 nM). The magnitude of the chemotactic response was greater for the fragment than for the whole molecule (by 15-50%).

Both types of motility were sensitive to inhibition by GRGDS but not to the inactive analogue GRGES. In fact, the motility stimulated by the 33 kDa fragment was more profoundly inhibited by GRGDS than that stimulated by whole fibronectin (with only ~20% of control motility evoked in the presence of 200-300 nM peptide). As was demonstrated for whole fibronectin, motility stimulated by the 33 kDa fragment was unaffected by pertussis toxin, cholera toxin, forskolin, or cell permeable cAMP analogues such as 8-Bromo-cAMP.

Publications

Stracke ML, Aznavoorian SA, Beckner ME, Liotta LA, Schiffmann E. Cell motility, a principal requirement for metastasis. First International Conference on Cytokines and Cell Motility. Basel, Switzerland: Birkhauser Verlag, 1991;147-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201 CB 00892-08 LP
PERIOD COVERED October 1, 1990 to September 30, 1991		
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:	L. Liotta Chief, Tumor Invasion and Metastases Section	LP NCI
	N. MacDonald Visiting Fellow	LP NCI
	A. Leone Visiting Associate	LP NCI
	A. Golden Howard Hughes Research Scholar	LP NCI
	U. Flatow Biologist	LP NCI
	M. Benedict Biologist	LP NCI
COOPERATING UNITS (if any) Molecular Oncology Incorporated, Gaithersburg, MD (CRADA)		
LAB/BRANCH Laboratory of Pathology		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 5.25	PROFESSIONAL: 2.5	OTHER: 2.75
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
		B
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The genetic regulation of tumor metastasis was investigated. <i>Nm23</i> was identified on the basis of its reduced steady state RNA levels in highly metastatic murine melanoma cell lines. Reduced <i>nm23</i> steady state RNA levels were observed in a subset of human breast carcinoma patients; in two reports these patients exhibited greater numbers of lymph node metastases, and significantly reduced disease free and overall survival. Two human <i>nm23</i> genes were identified, <i>nm23-H1</i> and <i>nm23-H2</i>, both of which encode 17 kDa proteins.</p> <p><i>Nm23</i> has been demonstrated to have two characteristics of a cancer suppressor gene: Transfection of murine <i>nm23-1</i> into highly metastatic murine K-1735 TK melanoma cells resulted in a reduced incidence of primary tumor formation, significant reductions in tumor metastatic potential, and altered <i>in vitro</i> responsiveness to the cytokine TGF-B. <i>Nm23-H1</i> was mapped to 17q21 in the C.E.P.H. database, and its somatic allelic deletion was noted in human breast, lung, renal and colorectal carcinomas. A homozygous deletion of <i>nm23-H1</i> was also observed.</p> <p>Multiple regulatory mechanisms for <i>nm23</i> in human cancer have been discovered, in addition to its reduced expression. In colorectal carcinoma <i>nm23-H1</i> allelic deletion, and not overall protein/RNA expression, was significantly correlated with the development of distant metastases. In childhood neuroblastoma, preliminary data have correlated increased <i>nm23</i> RNA expression and gene amplification with high metastatic potential.</p> <p>The human <i>nm23-H1</i> and <i>nm23-H2</i> cDNAs have been subcloned into an expression construct, and human breast and ovarian carcinoma cell lines are in the process of being transfected.</p> <p>The murine <i>nm23-1</i>, and human <i>nm23-H1</i> and <i>nm23-H2</i> cDNAs have been expressed in bacteria, and demonstrated to encode a nucleoside diphosphate kinase (NDPK). Comparison of tumor cell NDPK activity and <i>nm23</i> expression suggests that variations in <i>nm23</i> expression and the metastatic phenotype cause only minor changes in total NDPK activity. In order to determine the relationship between NDPK function and biological suppression of tumor incidence and metastasis, eleven site directed mutant <i>nm23</i> constructs were prepared. The NDPK activity of the mutated <i>nm23-1</i> cDNAs will be determined by bacterial expression, and metastasis regulatory activity by transfection into K-1735 TK melanoma cells.</p>		

Major Findings:

My laboratory has investigated the genetic regulation of the tumor metastatic process. I discovered the *nm23* gene on the basis of its reduced steady state RNA levels in highly metastatic murine K-1735 melanoma cells. Subsequently, reduced *nm23* steady state RNA levels were reported in tumor cells of high metastatic potential of three rodent metastasis model systems. In human breast cancer, reduced *nm23* steady state RNA levels were associated with the presence of lymph node metastases, and significantly reduced patient disease free and overall survival. Two independently regulated human *nm23* genes were discovered, *nm23-H1* and *nm23-H2*.

Considerable effort during the current year was directed toward the completion of studies that determined whether *nm23* was a suppressor gene. We reported that transfection of murine *nm23-1* cDNA into highly metastatic murine K-1735 TK melanoma cells resulted in a reduced incidence of primary tumor formation, and significant reductions in tumor metastatic potential. In addition, we reported the somatic allelic deletion of human *nm23-H1* in human breast, non-small cell lung, renal and colorectal carcinomas; the paired normal-tumor DNA samples were examined for evidence of allelic deletion at other chromosome 17 loci, and in 3/9 informative cases, *nm23-H1* allelic deletion occurred in the absence of other detectable allelic deletion events. A homozygous *nm23-H1* deletion was detected in a lymph node metastasis of a colorectal carcinoma.

The past year has also been one of initiating new *nm23* projects. Three major long-term questions are under investigation: (a) How is *nm23* regulated in various tumor cell types, and what is its relationship to metastatic behavior? (b) What is the biological effect(s) of transfection of the human *nm23-H1* and *nm23-H2* genes into those human tumor cells where reduced expression correlates with metastatic behavior? (c) What biochemical function(s) is responsible for the *nm23* suppressive effects?

Biochemistry

An independent report noted a 60% amino acid identity between *nm23* and *Dictyostelium* nucleoside diphosphate kinases (NDPK). In collaboration with Dr. Allen Shearn, Johns Hopkins University, we positively identified the *Drosophila* homologue of *nm23*, *awd*, to be a microtubule associated NDPK. In collaboration with Dr. Richard Kahn, LBC, DCT, NIH, the murine *nm23-1*, and human *nm23-H1* and *nm23-H2* proteins were expressed in bacteria, and the lysates demonstrated to have potent NDPK activity. Further purification and characterization is planned.

Besides the NDPK activity, additional interesting amino acid sequences have been identified on *nm23* proteins that suggest other biochemical functions. A leucine repeat suggestive of a leucine zipper is present in all higher vertebrate *nm23/awd/NDPK* sequences, and some *nm23* proteins contain an unpaired cysteine and/or a protein kinase C substrate sequence. In order to determine which, if any, of these functions accounts for the suppressive effects of *nm23-1* in murine K-1735 TK melanoma cells, we have undertaken site directed mutagenesis experiments. To date, eleven constructs have been prepared, each of which has mutated one or more amino acids encoded by the murine *nm23-1* cDNA sequence.

These cDNA clones will be expressed in bacteria, to determine the effect of each mutation on NDPK activity, leucine zipper dimerization and protein kinase C activity. The constructs will then be transfected into K-1735 TK melanoma cells, to ask if they suppress primary tumor incidence and metastatic potential.

Given the fact that the *nm23* cDNA clones encode NDPK, we have asked whether total cellular NDPK activity correlated with tumor metastatic potential. In transfected cell lines *nm23* steady state RNA levels were compared to NDPK activity, measured by both a radioisotopic and a coupled enzyme spectrophotometric assay. In general, variations in NDPK activity were a small fraction of the variation observed in *nm23* steady state RNA levels, either in whole cell lysates or fractionated cells. Our current hypothesis is that multiple NDPK genes exist, which mask the effects of altered *nm23*-NDPK levels. The data indicate that minor differences in total NDPK activity were correlated with significant alterations in K-1735 TK melanoma tumor incidence and metastatic potential and argue that, if NDPK activity is responsible for the biological suppressive effect, specific mechanisms must be involved.

Nm23 Expression-Breast Cancer

An independent report correlated reduced *nm23* steady state RNA levels with significantly decreased patient disease free and overall survival. In collaboration with Dr. Robert Barnes, University of Florida at Gainesville, a limited infiltrating ductal breast carcinoma cohort was stained with anti-*nm23* peptide 11 antibody. The staining was evaluated by three pathologists independently, and all identified a subgroup of patients with homogeneous or focal low staining tumor cells that had decreased overall survival. *Nm23* was an independent predictor in Cox's regression model when evaluated by two pathologists, and approached significance ($\alpha = 0.06$) by the third. A large scale retrospective analysis of *nm23* in node-negative breast cancer is being conducted with Dr. Dennis Slamon, UCLA.

Western blots containing lysates of bacteria expressing the *nm23-H1* and *nm23-H2* proteins indicate that anti-*nm23* peptide 11 antibody used in the initial prognostic studies cross reacted to both proteins. We have identified a region of the *nm23-H1* and *nm23-H2* proteins that is 60% different, and affinity purified antibodies to synthetic peptides corresponding to these regions have been prepared. The specificity of these antibodies is under characterization. These antibodies may elucidate the different regulation of the *nm23-H1* and *nm23-H2* genes, and may improve prognostic studies.

We are asking how *nm23* expression is decreased in highly metastatic breast carcinoma cells. In collaboration with Dr. Marc Lippman, Georgetown University, we have obtained preliminary evidence that estrogen decreased the *nm23* steady state RNA levels of estrogen-responsive breast carcinoma cells both *in vitro* and *in vivo*. In addition, in collaboration with Dr. Dennis Slamon, UCLA, we have obtained preliminary evidence that *neu* oncogene overexpression in human MCF-7 breast carcinoma cells also decreases *nm23* steady state RNA expression.

Nm23 Expression in Other Cancers

In cancer types other than breast carcinoma, alterations in *nm23*, but not its reduced expression have been associated with tumor metastatic potential. *Nm23* steady state RNA levels were elevated in colonic polyps and carcinomas of Dukes' Stages A-D, as compared to adjacent normal colonic epithelium. However, at the DNA level we observed a progressive series of allelic deletions in a single patient, from an allelic deletion in the primary carcinoma to a homozygous deletion in the lymph node metastasis. Based on this progressive DNA alteration with metastasis, *nm23-H1* allelic deletion was determined in a cohort of colorectal carcinoma patients with no evidence of distant metastases at surgery, in collaboration with Dr. Kenneth Cohn, SUNY, Brooklyn Health Science Center. Of patients with *nm23-H1* allelic deletions, 78% developed distant metastases over a mean followup period of 24 months, while only 20% of patients without *nm23-H1* allelic deletions did so. Planned studies will investigate whether mutations accompany allelic deletion in colorectal carcinoma.

In collaboration with Drs. Dennis Slamon, UCLA and Robert Seeger, USC, steady state *nm23* RNA levels in childhood neuroblastoma were found to increase with metastatic potential, independently of *myc* amplification. Southern blots indicate that increased expression is due to amplification of one allele of *nm23-H1*. Current experiments are determining whether *nm23* is mutated in high stage neuroblastoma. This "reverse" correlation of *nm23* expression and metastasis is intriguing, given the unique characteristics of neuroblastoma: Aneuploidy is also a "reverse" prognostic factor for this disease. In addition, a clear link between clinical course and cell differentiation can be observed in neuroblastoma. This system may provide unique models to sort out the biochemistry of *nm23*.

Transfections

The full-length human *nm23-H1* and *nm23-H2* cDNAs were subcloned into a constitutive expression vector, directed by a CMV promoter. Two cell lines were selected for analysis. The MDA passage of MDA-MB-435 breast carcinoma cell lines has low levels of *nm23-H1* and, to a lesser extent *nm23-H2*, as compared to the tumorigenic but nonmetastatic MCF-7 breast carcinoma cell line. It has been reported to metastasize in nude mice. The human ovarian carcinoma OVCAR3 cell line has also been reported to colonize the peritoneum of nude mice, and develop lung micrometastases. Transfections of these cell lines are underway. Bulk transfectants of *nm23-H1* and *nm23-H2* in both cell lines have been obtained, which express an exogenous *nm23* mRNA on Northern blots and synthesize increased amounts of immunoprecipitable *nm23* protein. *In vitro* and *in vivo* characteristics of these transfectants, as well as individual clonal lines, will be characterized.

Genomic Structure

In order to study the regulation of *nm23* transcription in metastasis and development, 19 clones were identified from a human genomic library. On Southern blots, one clone hybridized under stringent conditions to a *nm23-H2* probe, three clones hybridized to a *nm23-H1* probe, and the remainder may encode related genes and/or pseudogenes.

Additional Metastasis Related Genes

In collaboration with Drs. Vivian Zabrenetzky and David Roberts, LP, DCBDC, NCI, NIH, the steady state levels of thrombospondin were determined in human and rodent tumor metastasis model systems. In general, highly metastatic tumor cells exhibited reduced thrombospondin steady state RNA levels. These data are consistent with the hypothesis that thrombospondin may inhibit the angiogenic process, and suggest the potential prognostic importance of this gene. The thrombospondin cDNA is being subcloned into a CMV promoter driven expression construct for transfection experiments, to determine the effect of thrombospondin production on tumor cell angiogenesis and metastatic potential.

Publications:

- Liotta LA, Steeg PS. Clues to the function of Nm23 and Awd proteins in development, signal transduction, and tumor metastasis provided by studies of *Dictyostelium discoideum*. *J Natl Cancer Inst* 1990;82:1170-2.
- Biggs J, Hersperger E, Steeg PS, Liotta LA, Shearn A. A *Drosophila* gene that is homologous to a mammalian gene associated with tumor metastasis codes for a nucleoside diphosphate kinase. *Cell* 1990;63:933-40.
- Stahl J, Leone A, Rosengard AM, Porter L, King CR, Steeg PS. Identification of a second human *nm23* gene, *nm23-H2*. *Cancer Res* 1991;51:445-9.
- Leone A, Flatow U, King CR, Sandeen MA, Margulies IMK, Liotta LA, Steeg PS. Reduced tumor incidence, metastatic potential, and cytokine responsiveness of *nm23*-transfected melanoma cells. *Cell* 1991;65:25-35.
- Leone A, McBride OW, Weston A, Wang MG, Anglard P, Cropp CS, Goepel JR, idereau R, Callahan R, Linehan WM, Rees RC, Harris CC, Liotta LA, Steeg PS. Somatic allelic deletion of *nm23* in human cancer. *Cancer Res* 1991;51:2490-3.
- Hart M, Steeg PS, Willson JKV, Markowitz SD. NM23 gene expression is induced in human colonic neoplasms and is equal in colon tumors of both high and low metastatic potential. *J Natl Cancer Inst* (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09131-07 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	V. Cioce	Guest Researcher	LP NCI
	A. Claysmith	Biologist	LP NCI
	K. Barker	Guest Researcher	LP NCI
	E. Campo	Guest Researcher	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:

4.0

PROFESSIONAL:

2.7

OTHER:

1.3

CHECK APPROPRIATE BOX(ES)

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

B

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

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 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. Recently, we have shown that expression of both the laminin receptor protein and the mRNA are significantly increased in human colorectal carcinomas. In addition to its ability to bind to the carbohydrate structures on laminin, the 67 kDa laminin receptor apparently also has the ability to bind to specific amino acid sequences on the laminin molecule. We have recently established that the 67 kDa laminin receptor is synthesized from a cytoplasmic precursor with an approximate molecular mass of 37 kDa. We recently purified two other nonintegrin laminin binding proteins, HLBP31 and HLBP12, from both human placenta and human cancer cell lines. HLBP31 and HLBP12 have apparent molecular masses of 31 kDa and 12 kDa, respectively. Protein microsequencing identified HLBP31 as a previously characterized protein that has been called a beta-galactoside binding lectin, the low affinity IgE binding protein, or Mac-2 by different groups. HLBP12 has been previously identified as a soluble beta-galactoside binding lectin. We isolated a cDNA clone of HLBP31. The level of HLBP31 mRNA is inversely modulated with the 67 kDa laminin receptor in human colorectal carcinomas. 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:Biosynthesis of the 67 kDa laminin receptor

We previously identified a cDNA clone encoding the putative precursor of the 67 kDa laminin receptor. The precursor was identified in cell extracts as a 37 kDa polypeptide. Using affinity purified antibodies directed against cDNA-deduced synthetic peptides of the precursor in pulse chase experiments, we demonstrated a precursor-product relationship between the precursor and the 67 kDa laminin receptor. In transfection experiments using COS cells, we showed that transient transfection of the full-length precursor of the laminin receptor induced a dramatic increase in the synthesis of the precursor but not of the mature 67 kDa receptor, suggesting that the 67 kDa laminin receptor results from the association of two different gene products: the previously characterized 37 kDa precursor and another polypeptide that is yet to be identified. We predict that the additional polypeptide confers lectin-binding properties to the 67 kDa laminin receptor and shares a common epitope with HLBP31 and HLBP12.

Identification of two additional nonintegrin laminin binding proteins

We identified and purified two additional laminin binding proteins, designated HLBP31 and HLBP12, 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 low affinity 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. HLBP12 has a molecular mass of 12 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 HLBP12. These antibodies also recognize the 67 kDa laminin receptor on immunoblots and in immunoprecipitation experiments, demonstrating that HLBP31, HLBP12, and the 67 kDa laminin receptor share a common epitope and possibly a similar mechanism of laminin interaction.

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 67 kDa laminin receptor 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 23 individual patients with colorectal carcinomas for their steady state levels of laminin receptor mRNA and HLBP31 RNA. In 22 out of 23 patients with colon cancer, we found a 2- to 23-fold increase in the levels of 67 kDa laminin receptor mRNA in the cancer tissues compared with those in matched normal adjacent colonic mucosa. In contrast, steady state levels of HLBP31 mRNA were down regulated in 21 colon carcinoma lesions compared to their corresponding normal colonic mucosa. Furthermore, both the steady state levels of HLBP31 mRNA and the ratio of HLBP31/67 kDa laminin receptor mRNA in the primary colon carcinoma lesions were significantly ($p < 0.01$ and $p < 0.05$, respectively) lower in metastatic versus nonmetastatic colon carcinoma. Since the mRNA level of HLBP31 correlates with the amount of HLBP31 protein expressed in various human cancer cells, the data suggest that HLBP31 is down regulated in colon carcinoma. The degree of down regulation increased concurrently with the development of the metastatic phenotype. The data suggest that the determination of the relative mRNA levels of HLBP31 and the 67 kDa laminin receptor 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 67 kDa laminin receptor to use in *in situ* hybridization of cytospin and tissue sections. There is an increased amount of *in situ* hybridizable mRNA for the 67 kDa laminin receptor 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:

Cioce V, Castronovo V, Shmookler BM, Garbisa S, Grigioni WF, Liotta LA, Sobel ME. Increased expression of the laminin receptor in human colon cancer. *J Natl Cancer Inst* 1991;83:29-36.

Sobel ME, Liotta LA. Receptor-ligand interactions: role in cancer invasion and metastasis. In: Bellve AR, Vogel HJ, eds. *Molecular mechanisms in cellular growth and differentiation*. San Diego: Academic Press, 1991;107-20.

Hendrix MJC, Wood WR, Seftor EA, Lotan D, Nakajima M, Misirowski RL, Seftor REB, Stetler-Stevenson WG, Bevacqua SJ, Liotta LA, Sobel ME, Raz A, Lotan R. Retinoic acid inhibition of human melanoma cell invasion through a reconstituted basement membrane and its relation to decreases in the expression of proteolytic enzymes and motility factor receptor. *Cancer Res* 1990;50:4121-30.

Fernandez MT, Castronovo V, Rao CN, Sobel ME. The high affinity murine laminin receptor is a member of a multicopy gene family. *Biochem Biophys Res Commun* 1991;175:84-90.

D'Errico A, Garbisa S, Liotta LA, Castronovo V, Stetler-Stevenson WG, Grigioni WF. Augmentation of type IV collagenase, laminin receptor, and Ki67 proliferation antigen associated with human colon, gastric, and breast carcinoma progression. *Modern Pathol* 1991;4:239-46.

Castronovo V, Claysmith AP, Barker KT, Cioce V, Kruttsch H, Sobel ME. Biosynthesis of the 67 kDa high affinity laminin receptor. *Biochem Biophys Res Commun* 1991;177:177-83.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09163-04 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Anticancer Effects of a Novel Drug, CAI (NSC 609974) [Merck, L651582]

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:	L. Travers	Guest Researcher	LP, NCI
	W. Jacobs	Guest Researcher	LP, NC
	E. Schiffmann	Scientist Emeritus	LP, NCI
	L. Liotta	Chief, Tumor Invasion and Metastases Section	LP, NCI

COOPERATING UNITS (if any)

G. Curt, Associate Director, DCT, NCI; M. Greever, Director, Developmental Therapeutics Program, DCT; M.A. Sandeen, Research Technician, FCRDC, DCBDC Animal Holding Unit; C. Felder, Senior Staff Fellow, Lab. of Cell Biol., NIMH

LAB/BRANCH

Laboratory of Pathology

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.9

PROFESSIONAL:

1.4

OTHER:

1.5

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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.)

During the past year, we have made strides in understanding *in vitro* and *in vivo* effects of Merck L651582, now called CAI for carboxyamido-imidazole [NSC 609974D]. In collaboration with Dr. Chris Felder of the NIMH, we have demonstrated that CAI inhibits receptor-gated calcium influx and arachidonic acid metabolism in muscarinic receptor-transfected CHO cells. This inhibition was dose dependent and was within the concentration range that we have shown previously to inhibit AMF-stimulated inositol phosphate production. We have initiated studies to identify potential CAI effects against other components of the metastatic cascade and have shown that CAI inhibits type IV collagenase production and coordinately, TIMP-2 production. Animal studies have yielded new results on efficacy and preclinical pharmacology. We have demonstrated inhibition of OVCAR-3 tumor progression and metastasis and inhibition of A2058 human melanoma tumor incidence and growth in experimental animals, both with orally delivered CAI. Oral administration results in plasma levels in mice, rats, and dogs that are within the range of concentrations which *in vitro* inhibit signal transduction, growth, and motility [1-5 µg/ml]. CAI has passed into phase IIb preclinical development and is presently undergoing INDA-directed toxicity studies with intent to initiate Phase I clinical trials within the calendar year 1991. In addition, the patent application submitted in 1989 has had claims approved for use as an anticancer agent for ovarian cancer; further claims remain under review.

Major Findings:

We have been studying a novel antiproliferative and antimetastatic agent, the carboxyamido-imidazole, CAI, developed by Merck Institute for Therapeutic Research (Rahway, N.J.). This compound inhibits steps in receptor-mediated signal transduction pathways.

We have shown previously that this agent decreases autocrine motility factor (AMF)- and laminin-stimulated tumor cell motility, inhibits tumor cell adhesion to tissue culture plastic and laminin substrata, decreases AMF-stimulated phosphatidyl inositol metabolism that produces inositol trisphosphate, inhibits tumor cell proliferation of a wide variety of tumor cell lines *in vitro*, and prolongs survival of human ovarian cancer OVCAR3-bearing mice. Also, we have evaluated potential analogs in structure/function studies of CAI (described briefly below) and pursued preclinical development.

During this year, we have pursued the function, mode of action, and behavioral effects as well as the clinical utility of CAI in a number of ways: 1) continued study into its inhibitory effects on calcium fluxes and G-protein mediated signal transduction pathways; 2) investigation of its inhibition of tumorigenesis *in vitro* using soft agar colony-forming assays; 3) pursued structure/function studies with analog development; 4) identified antiproliferative and antimetastatic effects of orally administered CAI in two animal models and collaboratively evaluated the preclinical pharmacology of CAI; and 5) initiated studies in the inhibition of type IV collagenase production by CAI.

1. In collaboration with Dr. Christian Felder of the NIMH, we have pursued evaluation of the mechanism of signal transduction inhibition by CAI. M5 muscarinic receptor-transfected CHO cells were used for these studies; second messenger generation from muscarinic receptor stimulation by carbachol has been characterized completely by Dr. Felder for this cell line. CAI has a dose-dependent inhibitory effect upon carbachol-mediated arachidonic acid release and receptor-gated calcium influx. These effects are in the 1 - 10 μM range. Studies are planned to further characterize the effects of CAI on receptor-gated calcium influx and intracellular calcium changes. In addition, we are investigating endogenously expressed muscarinic receptors which we have demonstrated on the A2058 cells. Clarification of the signal transduction pathways mediating this muscarinic receptor activation is underway. We then propose to use this system to further characterize the mechanism of action of CAI.
2. We have developed colony-forming assays in soft agar for use with A2058 melanoma, CHOM5, and OVCAR3 cells. Inclusion of CAI in concentrations from 0.2 - 20 μM resulted in marked inhibition of colony initiation and colony growth. Other studies have demonstrated that TGF β also inhibits colony formation, whereas cholera and pertussis toxins increase colony number and size. Studies are ongoing to identify the signalling mechanism by which TGF β causes inhibition of colony formation and to understand how the bacterial toxins work in this system, which will include mixing studies with CAI in agar and in signalling studies.

3. Further studies of the analogs made during the summer of 1990 were unsuccessful. Repeat preparations did not yield reproducible results. Present plans are to pursue structure/function of CAI under contract to Dr. Richard Freer, a medicinal chemist and pharmacologist who will oversee two aspects of this project: 1) computer modeling of the structure to identify areas which can be modified, and 2) synthesis of modified chemical structures of CAI which will then be evaluated in our laboratory for their signal transduction, and growth and metastasis inhibitory properties. This is planned as an initiating step to studying the site of action of CAI in the cell. Other aspects of this project will include subcellular localization, preliminary to determining binding sites for CAI in the cell.

4. In collaboration with the Developmental Therapeutics Program, we have identified an optimal formulation for oral administration of CAI and have demonstrated that this formulation is effective *in vivo*. CAI can be solubilized in stable form in PEG400 at concentrations up to 150 mg/ml and can be administered to animals in this formulation. Oral dosing of nude mice at 100 mg/kg/d and 250 mg/kg/d yielded plasma levels over 2 µg/ml (~2-10 µM), levels which correlate with the inhibition of signal transduction *in vitro*. These levels result in an inhibition of tumor take from subcutaneously inoculated A2058 human melanoma as well as an arrest of tumor growth. Histologic evaluation of necropsy specimens showed tumor necrosis with an inflammatory infiltrate. Similar effects were seen when oral CAI was used in treatment of OVCAR3 human ovarian cancer-bearing mice. CAI slowed time to development of ascites, slowed tumor growth, and inhibited liver and lung parenchymal metastases. Observation of the animals and controls and necropsy evaluations did not suggest overt toxicity of CAI or vehicle. This work is being prepared for submission presently.

Preliminary toxicity studies done under Decision Network level IIB (INDA-directed toxicity studies) have not identified an LD10 in rats nor has significant toxicity been seen yet in dogs. Presently, major toxicity has been attributed to the intravenous formulation (cremophor/EtOH). Anemia and irritation has been seen at the higher doses given orally to the dogs.

It is presently anticipated that we will write the INDA and Phase I clinical trial in collaboration with CTEP, DCT and Dr. Greg Curt, respectively, with intent to initiate clinical trials of oral CAI within the calendar year 1991.

5. We have initiated investigation into applications of CAI effects. Dr. Peter Brown (Dr. Stetler-Stevenson's group) demonstrated that secretion and expression of the 72 kDa type IV collagenase was increased by TGF β and decreased by treatment with phorbol esters. We have confirmed those observations and have demonstrated that CAI inhibits baseline production and TGF β -induced production of the 72 kDa type IV collagenase at 2 μ M concentration. Further studies have shown that cellular treatment with 2 and 20 μ M CAI was associated with a marked decrease expression of the gene for the 72 kDa collagenase with little or no effect against expression of laminin receptor or nm23. Expression and production of TIMP-2 do not appear to be affected.

Publications:

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 Pharm Exp Therapeut (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09164-04 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Senior Staff Fellow	LP NCI
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	A. Levy	Microbiologist	LP NCI
	P. Brown	General Fellow	LP NCI
	A. Murphy	General Fellow	LP NCI
	E. Unsworth	Guest Researcher	LP NCI
	M. Gallagher	Student Volunteer	LP NCI

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Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

3.0

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

B

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

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. We have studied the transcriptional regulation of the 72 kDa collagenase IV enzyme in both normal and human tumor cell lines as well as human tumor tissues. 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 TGF β 1, are unaffected by the tumor promoting phorbol esters, and show elevated levels in colorectal tumor tissues when compared with adjacent normal mucosa tissues. Transfection of human tumor cells with expression constructs containing the 72 kDa type IV collagenase gene resulted in phenotypic changes, such as slowed cell growth rates and marked cell vacuolization. These findings suggest that uncomplexed 72 kDa type IV collagenase is not readily secreted by these transformed cells. 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 also examined the autoproteolytic breakdown of the 72 kDa type IV collagenase following removal of TIMP-2 from the complex. The enzyme has a characteristic pattern of autoproteolytic digestion generating specific 42 kDa and 37.5 kDa gelatin binding fragments, one (37.5 kDa) of which retains proteolytic activity. Finally, we have prepared antibodies and cDNA probes to other members of the collagenase family and have initiated a screening project to examine human lung tumor tissues for the relative mRNA levels and protein expression of the 72 kDa type IV collagenase, stromelysin-1, stromelysin-2, stromelysin-3, PUMP-1 and the 92 kDa type IV collagenase.

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. The cellular activation mechanism for the 72 kDa type IV collagenase appears cell surface associated and is inhibited by metalloproteinase inhibitors.
6. The cellular activation mechanism is specific for the 72 kDa type IV collagenase enzyme.
7. The cellular activation mechanism can be induced by specific treatments in various cell lines.
8. The 72 kDa type IV collagenase is secreted as a complex with TIMP-2.
9. Removal of the TIMP-2 from the 72 kDa type IV collagenase complex results in autoproteolytic degradation which generates characteristic 42 kDa and 37.5 kDa gelatin binding fragments.
10. The 37.5 kDa fragment retains proteolytic activity.
11. The 72 kDa type IV collagenase mRNA is induced following TGF β treatment.

Publications:

Brown, PD, Levy AT, Margulies IMK, Liotta LA, Stetler-Stevenson WG. Independent expression and cellular processing of M_r 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. *Cancer Res* 1990;50:6184-91.

Templeton NS, Brown PD, Levy AT, Margulies IMK, Liotta LA, Stetler-Stevenson WG. Cloning and characterization of human tumor cell interstitial collagenase. *Cancer Res* 1990; 50:5431-7.

Liotta LA, Stetler-Stevenson WG, Steeg PS. Metastasis suppressor genes. In: DeVita VT Jr, Hellman S, Rosenberg SA, eds. *Important advances in oncology* 1991. (in press)

Levy AT, Cioce V, Sobel ME, Garbisa S, Grigioni WF, Liotta LA, Stetler-Stevenson WG. Increased expression of the M_r 72,000 type IV collagenase in human colonic adenocarcinoma. *Cancer Res* 1991;51:439-44.

D'Errico A, Garbisa S, Liotta LA, Castronovo V, Stetler-Stevenson WG, Grigioni WF. Augmentation of type IV collagenase, laminin receptor, and Ki67 proliferation antigen associated with human colon, gastric, and breast carcinoma progression. *Mod Pathol* 1991;4:239-46.

Stetler-Stevenson WG. Type IV collagenases in tumor invasion and metastasis. In: Kerbel R, Frost P, Grieg E, eds. *Cancer and metastasis reviews*, vol 9. The Netherlands: Kluwer Academic Publ, 1990;289-303.

Grigioni WF, Garbisa S, D'Errico A, Baccarini P, Stetler-Stevenson WG, Liotta LA, Mancini AM. Evaluation of hepatocellular carcinoma aggressiveness by a panel of ECM antigens. *Am J Pathol* 1991;138:647-54.

Stetler-Stevenson WG, Krutzsch HC, Margulies, IMK, Liotta LA. Inhibition of human type IV collagenase by a highly conserved peptide sequence derived from its prosegment. *Am J Med Sci* (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09179-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Senior Staff Fellow	LP NCI
OTHER:	J. Ray	Staff Fellow	LP NCI
	A. Murphy	General Fellow	LP NCI
	G. D'Orazi	Visiting Fellow	LP NCI
	D. Kleiner	Medical Staff Fellow	LP NCI
	A. Levy	Microbiologist	LP NCI
	E. Unsworth	Guest Researcher	LP NCI

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

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. TGF β treatment of these cells results in a decrease in the TIMP-2 mRNA transcript levels. Along with induction of the 72 kDa type IV collagenase mRNA, this results in a shift in the enzyme inhibitor ratio in favor of proteolysis. These studies have also 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 and that the mechanism for this effect is through inhibition of endothelial cell proliferation. Finally, we have shown that TIMP-2 inhibits tumor cell invasion through reconstituted basement membranes *in vitro*.

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.

Publications:

Stetler-Stevenson WG, Brown PD, Onisto M, Levy AT, Liotta LA. Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. *J Biol Chem* 1990;265:13933-8.

Liotta LA, Stetler-Stevenson WG, Steeg PS. Invasion and metastasis. In: Holland JF, ed. *Cancer medicine* (in press)

Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. *Cell* 1991;64:327-36.

Albini A, Melchiori A, Parodi S, Liotta LA, Brown PD, Stetler-Stevenson WG. TIMP-2 inhibits tumor cell invasion. *J Natl Cancer Inst* (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09185-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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Laboratory of Pathology

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Tumor Invasion and Metastases Section

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TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

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

B

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

The signal transduction pathways and second messengers which cause a tumor cell to go from a resting to a migrating state are largely unknown. Previous studies with the human melanoma cell line A2058 have shown that the transduction of signals initiating motility differed, depending on the attractant and, in the case of certain extracellular matrix molecules, on whether the attractant was in solution or bound to a substratum. For example, pretreatment of these cells with pertussis toxin completely abolishes their motility response to their own autocrine motility factor and to type IV collagen in solution, but not to insulin-like growth factor (IGF-1), or to type IV collagen which is substratum-bound. Sensitivity to PT implicates the involvement of one of the transducer G proteins in these particular biochemical pathways, although its precise identity and mode of action is as yet unknown. Cloning of the gene in A2058 cells which encodes the relevant PT-sensitive G protein should provide answers to some of these questions. An A2058 cDNA library was screened with probes derived from various rat G protein clones. After a secondary screen, over 100 potentially positive clones were identified. The first 25 of these have been carried through a tertiary screen, with 17 still testing positive for one or more classes of G protein ($G\alpha_0$, $G\alpha_1$, and $G\alpha_2$ have been tentatively identified). These 17 are being prepared for sequencing and subcloning into antisense expression vectors, in order to identify which class of G protein is actually involved in motility. In addition, mRNA for $G\alpha_0$ and $G\alpha_2$, but not $G\alpha_1$, has been detected in A2058 cells. Western immunoblotting with an antisera specific for $G\alpha_0$ has detected this protein in membranes of A2058 cells; antisera specific for the various classes of G proteins will be used to determine which, if any, class of G proteins redistributes to pseudopodial protrusions during tumor cell migration.

Major Findings:

An A2058 cDNA library in λ gt11 was screened by plaque hybridization at low stringency with a mixture of probes to G protein α subunits. This initial probe, labeled by the random primer method, consisted of a mixture of full-length cDNAs coding for the α subunits of rat G_{i1} , G_{i2} , and G_o , and a 600 bp restriction fragment of the cDNA coding for the rat G_{i3} . (These cDNAs were originally isolated from rat olfactory neuroepithelium, and kindly provided by Dr. Randall Reed of Johns Hopkins University). $\sim 1 \times 10^6$ λ phage plaques were screened, and nearly 200 positive clones were obtained and collected in a buffer. A secondary screening at low stringency was then performed with a similar mixture of G protein probes. For this screen, dilutions of the phage supernatant from individual plaques were "spotted" directly on to a lawn of appropriate bacteria (10 μ l/spot), and plates were incubated until new plaques were formed. Filter transfers of phage plaques were then carried out as before, followed by a second round of hybridizations. 111 of the original clones were still positive after this secondary screening. As of this report, the first 25 of these have been taken through tertiary screenings sequentially with individual G protein probes (as opposed to the mixture of probes). For the tertiary screen, the phase supernatant was plated onto lawn bacteria at a dilution which produced only 20-100 well isolated plaques per 100 cm^2 dish. Plaque hybridization was performed at low stringency with, sequentially, the probe for each class of G protein α subunit. In addition, restriction fragments of the cDNAs for G_{i1} and G_o produced class-specific probes for these G proteins, which were also used in the tertiary screen. Use of the restriction fragment probes thus allowed tentative identification of those clones encoding a specific class of G protein (whereas the full-length cDNA probes cross-reacted to some extent among the different classes). To date, 17/25 of the clones in the tertiary screen have been positive for at least one of the G protein clones; two have reacted positively with the G_o -specific probe, one has reacted positively with the G_{i1} -specific probe, and none have reacted with the G_{i3} -specific probe. For G_{i2} , there was no restriction fragment which produced a probe specific for this class of G protein; however, the strength of the hybridization signals given by certain clones to the full-length cDNA probe strongly suggests that several clones code for G_{i2} . Thus, in A2058 cells, it appears that more than one type of pertussis toxin-sensitive G protein exists; subcloning and sequencing the clones will allow definitive identification. For each of the 17 clones which tested positive, high-titer phage plate lysates were produced, and λ "minipreps" were performed with the Qiagen kit to isolate the recombinant phage DNA. cDNA inserts were then excised with ECO RI, and the digestion products were run on an agarose gel for a Southern hybridization with the individual G protein probes. This procedure allowed detection of the sizes of the individual G protein inserts; inserts will then be subcloned and sequenced.

To begin to answer the question of which of the several classes of G proteins in A2058 cells is actually involved in chemotaxis, Northern blot analysis was performed on cells which had been treated for 48 hr with various chemotactic stimuli. Concentrations of type IV collagen and laminin which maximally stimulate chemotaxis, and a crude AMF sample, were added to near-confluent cells in culture. RNA was extracted from these cells and from a parallel "control" culture (with no chemotactic stimulus) 48 hr later. Northern blot analysis was performed on the total RNA with each of the G protein probes, in order to determine the sizes of the messages for the various G proteins, and also to determine if the levels of messages for any G protein changed upon chemotactic stimulation (indicating a change in the level of the corresponding protein). With the G_o probe, 5 bands were present in autoradiographs: one major band at 4.4 Kb, and four minor bands at ~2.3 Kb, ~1.5 Kb, ~1.0, and ~0.5 Kb. With the probe for G_{i2} , one band was detected at ~2.3 Kb; no message was detected with the probes for G_{i1} and G_{i3} , even after long exposure times (ie, 1 week). This experiment was then repeated using only type IV collagen as the chemotactic stimulus, and RNA was extracted after incubation times of 4 hr, 24 hr, and 48 hr. Northern blot analysis with the same probes detected messages of the same sizes for G_o and G_{i2} as in the first experiment; however, no reproducible changes in message levels for either class of G protein were observed upon chemotactic stimulation, relative to control levels.

Since these preliminary experiments suggest that the level of expression of the relevant G protein(s) does not change upon chemotactic stimulation, it is conceivable that a redistribution of G proteins to the leading edge (or pseudopod) of a migrating cell occurs. A recent report (Bourguignon et al, J. Immunol. 144:2242-2252, 1990) demonstrated that a lymphoma membrane-associated 41-kDa GTP-binding protein (a $G_{i\alpha}$ -like protein) is closely associated with several cytoskeletal proteins and is preferentially accumulated underneath receptor capped structures. An analogous situation could occur upon chemotactic stimulation of A2058 cells, in which receptors for the attractant (eg, type IV collagen) together with bound G protein, may migrate laterally in the cell membrane to the leading edge of the cell (pseudopod) which "senses" the attractant in solution. If this hypothesis is true, pseudopods from stimulated cells would be expected to contain a high concentration of the relevant G protein, compared to the rest of the cell body. Using standard chemotaxis chambers, and filters of 1 μ m pore size, pseudopods from type IV collagen- and AMF-stimulated cells will be isolated, collected, and analyzed by Western immunoblot analysis with class-specific antibodies to the various G proteins. Antisera are currently being generated against internal peptide sequences which distinguish G_{i1} , G_{i2} , G_{i3} , and G_o . These class-specific antisera will be useful in determining which type of G protein, if any, translocates with receptors to the pseudopodial protrusions of migrating cells. Additionally, these antisera will be used in immunofluorescence studies of migrated (whole) cells, in order to attempt to visualize the G protein redistribution to the pseudopods. A preliminary Western immunoblot was performed with a commercially available antisera specific for the α subunit of G_o ; using a sample of a crude membrane preparation from unstimulated cells, a single immunoreactive band was detected at ~39 kDa, strongly suggesting the presence of G_o in these cells.

A definitive method of identifying the G protein involved in A2058 motility will be employed which involves antisense RNA technology. This technique enables selective gene inhibition and provides stable "deletional" mutants. Self-replicating episomal replicons have recently been described and utilized as vectors for antisense RNA transfection (Groger, R.K. et al, Gene 81:285-294, 1989, and Hambor, J.E. et al, Proc. Natl. Acad. Sci. 85:4010-4014, 1988). Episomal replicons are circular DNA elements designed to self-propagate extrachromosomally to high copy numbers in eukaryotic cells. We have obtained these vectors, which are specifically engineered to alternatively permit directional antisense and sense cloning of cDNAs. A2058 cells will be transfected with these expression vectors into which the cDNAs corresponding to various G protein α subunits have been subcloned in an antisense orientation. This will serve the purpose of "shutting off" the expression of a particular class of G protein, so that chemotaxis to type IV collagen (and/or AMF) may be assessed under conditions of impaired G protein function.

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

PROJECT NUMBER

Z01 CB 09352-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Motility Regulator Proteins

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:

1.25

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.)

Monoclonal antibodies were screened for the ability to block the motility of human A2058 melanoma cells. A monoclonal antibody was identified which arrested tumor cell motility without altering adhesion. The antigen recognized by the antibody is a glycosylated cell surface protein with a molecular weight of 95 kDa. This antibody was used to screen a cDNA library. The cDNA clone isolated encodes a unique protein. Homology is noted with CD4, N-CAM and certain ion channel proteins. The predicted sequence has a transmembrane domain. Future plans include a structure-function analysis of this protein as a regulatory of motility and a correlative analysis of the levels of the protein or its mRNA associated with various stages of tumor progression.

Major Findings:

A new cell surface protein which regulates tumor cell motility has been identified. Monoclonal antibodies directed against this protein block motility and recognize a 95 kDa band on immunoblotting of cell lysates. This antibody was used to isolate a cDNA clone which encodes a novel protein with significant homology to the superfamily of N-CAM and CD4 cysteine repeat genes.

Future Studies:

Future long-term plans include the following:

complete structural and functional characterization of this protein, including potential augmentation or loss on motility associated pseudopodia, correlation with tumor progression, and relationship to cell-cell and cell substratum adhesion independent of motility.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09353-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	V. Castronovo	Visiting Scientist	LP NCI
OTHER:	M. Sobel	Senior Investigator	LP NCI
	F. van den Brule	Visiting Fellow	LP NCI

COOPERATING UNITS (if any)

Dr. F. Kridelka, University of Liege, Belgium; Dr. A. Berchuck, Duke University, North Carolina

LAB/BRANCH

Laboratory of Pathology

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Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

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- (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, endometrial, 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, a 31 kDa laminin binding protein with lectin binding properties, 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. Immunohistochemistry, Western immunoblot, Northern blot, and *in situ* hybridization techniques are being used to assess specific expression. 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 conducted to determine the specific effect of steroid hormones on human breast cancer cells. Breast cancer cell lines derived from steroid receptor-negative tumors express higher levels of 67 kDa laminin receptor than do steroid receptor-positive cell lines. Steroid receptor-positive tumor cells that are grown in the presence of estrogen and progesterone increase their expression of laminin receptor up to the levels of steroid receptor-negative cell lines.

Major Findings:Expression of the 67 kDa laminin receptor in breast cancer tissue

Matched normal and tumor breast samples from 16 patients are being studied. Patients were from the University of Liege, and all samples were freshly frozen at the time of surgery and stored for later analysis. Frozen sections of all samples have been made for immunohistochemical and *in situ* hybridization analysis. In addition, total protein extracts have been prepared and total cellular RNA has been isolated from each sample. To date, immunoblot analysis using anti-67 kDa laminin receptor antibodies shows an increased expression of the laminin receptor in the tumor versus matched normal breast tissue. Antibodies against a recombinant protein of the 31 kDa laminin binding protein also show an increased expression in the breast tumor tissue. Future immunoblot analysis is planned using anti-type IV collagenase and anti-TIMP-2 antibodies. Analysis of the total RNA samples has been hampered by degradation of RNA in most of the normal samples; however, the tumor samples have intact RNA. cDNA probes for the 67 kDa laminin receptor, 31 kDa laminin binding protein, type IV collagenase, and TIMP-2 will be used on Northern blots. *In situ* hybridization will be used as an adjunct to these studies, especially in patients who did not have sufficient intact total RNA for Northern blot analysis. Relative expression of the mRNAs for the target genes will be correlated with clinical course.

Preparation of human ovarian cancer samples for analysis

Eleven ovarian tumors and one normal ovary have been obtained from Duke University. Total protein extracts and intact total cellular RNA have been successfully prepared from all samples. Expression of the 67 kDa laminin receptor protein and mRNA are significantly increased in the ovarian cancer samples. Analysis of the expression of the other laminin binding proteins, collagenase, and TIMP is in progress. This study will be expanded with another 20 samples in the near future. All samples will be studied by immunohistochemical and *in situ* hybridization techniques as well.

Modulation of gene expression in human breast cancer cell lines by steroid hormones

We have previously shown that the expression of the 67 kDa laminin receptor mRNA is increased in the steroid receptor-negative human 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 these initial results by preparing new samples from MDA-MB231 and T47D cells, as well as the estrogen-responsive human breast cancer cell line MCF-7. Expression of the laminin receptor, 31 kDa laminin binding protein, type IV collagenase, and TIMP-2 is being studied in all cell lines grown in the absence and presence of steroid hormones. Both Western immunoblot and Northern and slot blot hybridization analysis is being carried out. The *in vitro* effects will be correlated with the functional difference in clinical aggressiveness between steroid receptor-negative and positive breast cancers.

Publications:

Castronovo V, Colin C, Claysmith AP, Chen PHS, Lifrange E, Lambotte R, Krutzsch H, Liotta LA, Sobel ME. Immunodetection of the metastasis-associated laminin receptor in human breast cancer cells obtained by fine-needle aspiration biopsy. *Am J Pathol* 1991;137:1373-8.

D'Errico A, Garbisa S, Liotta LA, Castronovo V, Stetler-Stevenson WG, Grigioni WF. Augmentation of type IV collagenase, laminin receptor, and Ki67 proliferation antigen associated with human colon, gastric, and breast carcinoma progression. *Modern Pathol* 1991;4:239-46.

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

PROJECT NUMBER

Z01 CB 00550-11 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Expert	LP NCI

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LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

3.0

OTHER:

1.0

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

A

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

In 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 new variant of T-cell lymphoma which appears to be a newly described clinicopathologic entity was reported. Eight patients were observed with a T-cell lymphoma involving subcutaneous tissue and mimicking panniculitis. This syndrome was highly associated with the development of hemophagocytosis. Six of the eight patients developed a florid hemophagocytic syndrome, which was fatal in five. Dissemination to non-subcutaneous sites did not occur. Three of the patients are currently alive without evidence of lymphoma after aggressive chemotherapy. Immunophenotypic analysis demonstrated a T-cell phenotype in all cases studied. Genotypic analysis demonstrated a rearrangement of the T-cell receptor beta chain gene in one (possibly two) of the three cases studied. These cases bear many similarities to the process reported as benign cytophagic panniculitis, and it is postulated that benign cytophagic panniculitis may represent a variant of T-cell lymphoma, or, alternatively, these cases represent the malignant counterpart of that entity.

Two patients with chronic lymphocytic leukemia and co-existent Hodgkin's disease were reported. The first patient was found to have Reed-Sternberg cells within lymph nodes at initial presentation of CLL, and he ultimately progressed to develop disseminated lymphoma characteristic of Hodgkin's disease. In the second patient, Reed-Sternberg cells developed five years after primary diagnosis of CLL. The Reed-Sternberg-like cells were associated with a background lymphocytic population characteristic of B-cell CLL. By contrast, the Reed-Sternberg cells were strongly positive for CD15 but usually negative for B-cell markers. In both patients, however, a small percentage of the Reed-Sternberg cells did express B-cell markers. These findings may indicate an origin for the Hodgkin's disease from the underlying CLL. Moreover, these findings support a B-cell origin for the malignant cell in some cases of Hodgkin's disease, and suggest that Hodgkin's disease in some patients may be related to, or derived from, a co-existing lymphoid malignancy.

The My4 antibody, one of a number of monoclonal antibodies reactive with the CD14 antigen, was originally reported to weakly stain monocytes, macrophages, and granulocytes. However, some studies have suggested that the My4 antibody may also stain both normal and neoplastic B cells. A series of 245 non-Hodgkin's lymphomas were stained with My4. Forty-five percent of cases (111) were positive, including 108 of 189 (57%) B-cell lymphomas. Three of 50 (6%) of T-cell lymphomas were positive. My4 positive B-cell lymphomas occurred in all histologic subtypes with the exception of small non-cleaved. Follicular lymphomas were most often My4 positive (82%). Other monoclonal antibodies reactive with the CD14 antigen were employed, but were consistently negative in My4 positive B-cell lymphomas. Thus, the staining of reactive and neoplastic B cells by My4 appears to be unique to this antibody, and is not a feature of all anti-CD14 antibodies.

A study was conducted to evaluate the utility of monoclonal antibodies reactive in routinely processed bone marrow specimens in the phenotypic analysis of acute lymphoblastic leukemia. Nineteen cases of ALL, which had previously undergone phenotypic and genotypic analysis, were studied.

The study concluded that monoclonal antibodies useful in the characterization of mature T and B cell lymphomas, are less useful when applied to lymphoblastic malignancies. Leu-22 stained both pre-B and T-cell ALLs, while UCHL-1 demonstrated artifactual nuclear staining of both forms of leukemia. L26 antibody reacted with only one case of pre-B ALL, while LN1 antibody was consistently negative in all cases. The LN2 antibody appeared to be most useful in that it stained 11 of 12 cases of pre-B ALL and did not react with any of the 7 T-ALLs studied.

Publications:

Jaffe ES, Raffeld M. Identification of cells in tissue sections; unit 5.8. In Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. Current protocols in immunology. New York: John Wiley & Sons; Greene Publishing Associates & Wiley Interscience, 1991.

Medeiros LJ, Jaffe ES. Pathology of malignant lymphomas. In Wiernick PH, Canellos GP, Kyle RA, Scheffer CA, eds. Neoplastic diseases of the blood, 2nd ed. New York: Churchill Livingstone, 1991;631-62.

Boumpas DT, Wheby MS, Jaffe ES, Steinberg AD, Klippel JH, Balow JE. Synovitis in angioimmunoblastic lymphadenopathy with dysproteinemia stimulating rheumatoid arthritis. Arth Rheum 1990;33:578-82.

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Lardelli P, Bookman MA, Sundeen J, Longo DL, Jaffe ES. Lymphocytic lymphoma of intermediate differentiation (IDL): Morphologic and immunophenotypic spectrum and clinical correlations. Am J Surg Pathol 1990;14:752-63.

Rushin JM, Riordan GP, Heaton RB, Sharpe RW, Cotelingam JD, Jaffe ES. Cytomegalovirus-infected cells express Leu-M1 antigen. A potential source of diagnostic error. Am J Pathol 1990;136:989-95.

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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. (in press)

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Kamel AM, Ghaleb FM, Assem MM, Hindawy, DS, Jaffe ES, Magrath IT. Phenotypic analysis of T-cell acute lymphoblastic leukemia in Egypt. Leuk Res 1990;14:601-9.

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Gonzalez CL, Medeiros LJ, Braziel RM, Jaffe ES. T-cell lymphoma involving subcutaneous tissue: A clinicopathologic entity commonly associated with hemophagocytic syndrome. Am J Surg Pathol 1991;15:17-27.

Pluda JM, Yarchoan R, Jaffe ES, Feuerstein IM, Solomon D, Steinberg SM, Marczyk KS, Raubitschek A, Katz D, Broder S. Development of opportunistic non-Hodgkin's lymphoma in a cohort of patients with severe HIV infection on long-term antiretroviral therapy. Ann Intern Med 1990;113:276-82.

Devaney K, Jaffe ES. The surgical pathology of gastrointestinal Hodgkin's disease. Am J Clin Pathol (in press)

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Taubenberger JK, Cole DE, Raffeld M, Poplack DG, Jaffe ES, Medeiros LJ. Immunophenotypic analysis of acute lymphoblastic leukemia using routinely processed bone marrow specimens. Arch Pathol Lab Med 1991;115:338-42.

Skopouli FN, Fox PC, Galanopoulou V, Atkinson JC, Jaffe ES, Moutsopoulos HM. T cell subpopulations in the labial minor salivary gland histopathologic lesion of Sjögren's syndrome. J Rheumatol 1991;18:210-4.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 00855-09 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Expert	LP NCI
	L. Roman	Medical Staff Fellow	LP NCI

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LAB/BRANCH

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SECTION

Hematopathology Section

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TOTAL MAN-YEARS:

0.20

PROFESSIONAL:

0.15

OTHER:

0.05

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

A

SUMMARY OF WORK (Use standard unrecuded 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:

A limited panel of monoclonal antibodies is available for immunophenotypic analysis in paraffin embedded material. For detection of T cells, the two most useful antibodies are UCHL-1 (CD45RO) and either Leu-22 or MT1 (CD43). In a pilot study of 25 cases of T-cell lymphoma from the Trinidad leukemia/lymphoma series, 23 of 24 cases tested were UCHL-1 positive and 20 of 23 were CD43 positive (Leu-22). There were four cases in which discordant results were obtained with UCHL-1 and Leu-22. Three of four were UCHL-1 positive/Leu-22 negative; one of four was UCHL-1 negative/Leu-22 positive. Of 23 cases tested for HTLV-1 associated antibodies, 21 were positive. Fourteen cases were identified as being of B-cell origin (L26 positive). Sera from only 1 B-cell patient was positive for HTLV-1 associated antibodies; this case was an 80 year old female with a large cell immunoblastic lymphoma that clinically did not appear to have ATLL.

Preliminary evidence suggests that EBV may be associated with angiocentric immunoproliferative lesions in many anatomic sites. Thus far, EBV has been identified either by PCR or by Southern blot in angiocentric immunoproliferative lesions obtained from lung and nasopharynx. Lesions involving skin, exclusively, have thus far been negative.

In a preliminary study of Kikuchi-Fujimoto disease, EBV was detected in a significant percent of lesions by the PCR technique. Other viruses are being investigated, and these observations are being pursued with *in situ* hybridization.

A patient with adult T-cell leukemia/lymphoma, being treated with anti-TAC monoclonal antibody, developed Kaposi's sarcoma. Following discontinuation of the monoclonal antibody therapy, there was spontaneous regression of the Kaposi's sarcoma lesion, as well as relapse of the ATLL.

Publications:

Greenberg SJ, Jaffe ES, Ehrlich GD, Korman NJ, Poesz BJ, Waldmann TA. Kaposi's sarcoma in human T-cell leukemia virus type I-associated adult T-cell leukemia. *Blood* 1990;76:971-6.

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

PROJECT NUMBER

Z01 CB 09181-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 Expert LP NCI

COOPERATING UNITS (if any)

Medicine Branch, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

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

A

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

We have continued our studies of the expression of bcl-2 in follicular lymphoma. We developed a procedure for isolation of RNA followed by reverse transcription and PCR amplification of bcl-2/heavy chain sequences. Using this method, we are able to measure the level of expression of bcl-2 in small specimens. We have compared our technique in cell lines carrying the t(14;18) with the standard procedure of Northern blot analysis and have documented the validity of our method. Using this procedure, we have measured the levels of expression of bcl-2 in a series of biopsy specimens. We are currently correlating our results with clinical outcome. Studies indicate that bcl-2 may incur a growth advantage to cells but that involvement of other oncogenes, especially myc, is necessary to attain full malignant potential. We have developed a similar technique utilizing PCR to measure myc transcripts and have evaluated the validity of this method using non-stimulated and PHA stimulated peripheral blood T cells and comparing our PCR results to Northern blot data. We are studying the expression of myc in the follicular lymphomas studied for bcl-2 expression. In addition, we are studying the expression of myc in lymphomas that have progressed to a more aggressive phenotype to determine if myc expression is associated with this phenotype. This will provide important information as to the interrelationship of various oncogenes in the development of follicular lymphoma.

Major Findings:

1. The level of expression of bcl-2 and myc can be determined by PCR and is comparable to Northern data.
2. The level of expression of bcl-2 differs among follicular lymphoma specimens and may relate to clinical prognosis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09182-03 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Biology of 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
	E. Jaffe	Chief, Hematopathology Section	LP NCI
	H. Hsieh	Guest Researcher	LP NCI
	H. Hollingsworth	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:

1.3

PROFESSIONAL:

1.3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

A, B

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

We have determined the frequency with which each of the breakpoint areas of bcl-2 are involved in translocation and are currently investigating whether the specific breakpoints might influence the clinical behavior of lymphoma. In the process, we are fine mapping the sites of these breakpoint regions.

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 and other tissue samples. We are continuing retrospective studies using fixed tissues.

We have shown the bcl-1 major breakpoint region is associated with malignant lymphoma of intermediate differentiation. We have examined several additional breakpoint regions and are currently initiating studies to investigate the role of the candidate bcl-1 gene, PRAD1.

We have identified and sequenced a T-cell receptor delta gene rearrangement occurring in over a third of precursor B ALL. This common rearrangement can be used to aid in the diagnosis of primary and particularly recurrent disease.

We have performed a molecular study of small non-cleaved cell lymphomas and have shown that molecular differences exist between the Burkitt's subgroup and the non-Burkitt's subgroup. The separation of these two entities has been controversial, and these results suggest the two subgroups have a different molecular pathogenesis, supporting the uniqueness of these entities.

We have initiated 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 angiocentric lymphomas, AILD, and Kikuchi's disease.

Major Findings:

1. PCR technology can be adapted to the diagnosis of follicular lymphoma with the t(14;18) translocation.
2. There is a fourth minor breakpoint cluster in the bcl-2 genetic locus on chromosome 18 and the pfl-2 (mcr) region consists of several distinct breakpoint miniclusters. The different clusters may have different clinical significance.
3. Precursor B cell ALL shows a high frequency of rearrangement of the T cell receptor delta gene locus involving V delta 2, D delta 2 and D delta 3. There is no coordinate J region joining. This finding can be used diagnostically.
4. Malignant lymphoma of intermediate differentiation shows a high frequency of rearrangement of the bcl-1 locus, implicating involvement of a nearby gene in its pathogenesis.
5. Undifferentiated malignant lymphomas of the Burkitt type almost uniformly show rearrangement of the c-myc gene but never involvement of bcl-2, while those of non-Burkitt's type occasionally show bcl-2 rearrangement, but rarely involvement of c-myc suggesting different pathogeneses for these morphologically similar subgroups.

Publications:

Medeiros LJ, Van Krieken JHJM, Jaffe ES, Raffeld M. Association of bcl-1 rearrangements with lymphocytic lymphoma of intermediate differentiation. Blood 1990;76:2086-90.

Raffeld M, Jaffe ES. Bcl-1, t(11;14) and mantle cell derived lymphomas. Blood (in press)

Yano T, Pullman AB, Raffeld M, Cossman J. A common V δ 1-D δ 2-D δ 3 T cell receptor gene rearrangement in precursor B acute lymphoblastic leukemia. Brit J Haematol (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09191-02 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Biology of 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
	A. Ginsberg	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:

1.7

PROFESSIONAL:

1.7

OTHER:

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.

As a first approach, we have studied a series of recurrent follicular lymphomas to assess the status of their immunoglobulin and bcl-2 restriction patterns over time. Changes in the bcl-2 gene restriction fragments were not observed. However, new immunoglobulin gene restriction fragments occurred frequently (30% of the cases studied). These new subclones must acquire a growth advantage to overgrow the original parent tumor. Thus, changes in the immunoglobulin gene restriction pattern serves as a marker for subsequent or coincident growth promoting mutations.

We have begun to investigate potential second events which could impart a selective growth advantage to a lymphoma cell which would manifest itself as histologic or clinical progression. To this end, we are currently analyzing the potential role of several oncogenes and anti-oncogenes in a large series of progressed lymphomas. We are studying the involvement of the c-myc gene and have found new rearrangements of this proto-oncogene in 10-15% of the progressed lymphomas. The molecular structure of c-myc rearrangement in these secondary high grade lymphomas appears to be different from that seen in primary high grade lymphomas and we are currently studying these differences. We have found retinoblastoma gene abnormalities at the DNA and RNA levels in several different types of lymphomas at a lower incidence. We have also surveyed a large series of lymphomas with probes for several other genetic loci associated with disease progression and/or high grade histology (bcl-3, bcl-4), but have found only sporadic involvement of these loci. We have recently initiated investigations into the role of some members of the rel gene family and p53.

Major Findings:

1. Immunoglobulin restriction patterns show frequent changes over time in follicular lymphomas and these changes can be used as markers for new subclones with growth advantages.
2. New c-myc gene rearrangements occur in a significant number of lymphomas undergoing histologic and clinical progression. The molecular structure of these rearrangements are different from those which commonly occur in high grade Burkitt's lymphomas.
3. Rb abnormalities occur occasionally in lymphoproliferative diseases, and seem to be associated with a more aggressive clinical course.
4. The previously reported progression related genes bcl-3 and bcl-4 are uncommonly associated with progression.

Publications:

Ginsberg AM, Raffeld M, Cossman J. Inactivation of the retinoblastoma gene in human lymphoid neoplasms. Blood 1991;77:833-40.

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.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09144-07 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	M. Takimoto	Guest Researcher	LP NCI
	M. Avigan	Medical Staff Fellow	LP NCI
	R. Duncan	IRTA 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:

3.75

PROFESSIONAL:

2.75

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.)

Application of an exonuclease assay developed in this laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc gene has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. We are presently focusing on 3 regions, each of which interacts with one or more sequence-specific binding proteins.

- 1.5 kb upstream of P1, a cell-type and differentiation specific 75 kD protein containing peptides, binds to a positive element.
- 100 to 150 bp upstream of P1, a complex set of factors bind to a cytidine-rich element shown to be a positive acting cis-element. Two factors binding to this positive element have been purified in this laboratory. A 60 kD polypeptide as well as a 32 kD species have been shown to interact independently with this c-myc element. Partial protein sequence has been obtained for each factor.
- Approximately 1 kb downstream, a 140 kD phosphoprotein binds to a negative element.

Identification of a c-myc far upstream stimulatory element (FUSE) regulated during differentiation

c-myc expression shuts off biphasically during DMSO-induced differentiation of HL-60 promonocytic leukemia cells. The first stage of c-myc down-regulation involves a block to transcriptional elongation at the end of exon 1 that is reversible upon withdrawal of the differentiating agent. The second phase is a shut down of transcriptional initiation that occurs later, is associated with changes in nuclease hypersensitive sites and is irreversible, correlating with commitment to differentiation. To identify cis- and trans-elements associated with these changes, extensive portions of c-myc 5' upstream sequence were subjected to exonuclease protection assays utilizing nuclear extracts prepared at different stages of differentiation. The disappearance of one particular factor coincided remarkably with the timing of shut off of transcriptional initiation and the alteration of c-myc chromatin. High resolution gels allowed the rapid and precise identification of the binding site of a factor present only in undifferentiated cells. An oligonucleotide corresponding to the binding site of this factor was prepared; using this probe, we confirmed the presence and regulation of this factor using gel retardation, UV-crosslinking and DNase I footprint analysis. We have constructed a CAT plasmid containing 3.2 kb of c-myc sequence including the far upstream element (FUSE) and the c-myc promoters fused to CAT. When transfected into U-937, HeLa, and Hep G2 cells, the plasmid expressed significant amounts of CAT. When a strategic 4 base deletion in the binding site of FUSE was made, there was a significant reduction of CAT expression in U-937 transfectants and HeLa cells but not Hep G2 transfectants, demonstrating that the element has a potentiating effect on expression when bound by the factor complex. Ion exchange, hydroxyapatite and oligonucleotide affinity chromatography of HL-60 leukemia cell extracts yield a prominent 75 kD polypeptide. Elution of this polypeptide from SDS-polyacrylamide gels followed by renaturation demonstrated the intrinsic FUSE binding activity of the 75 kD polypeptide. Cleavage of this polypeptide with cyanogen bromide and trypsin followed by preparative HPLC yields peptides. Edman degradation of these peptides allowed the synthesis of degenerate oligonucleotide primers. Utilization of these primers for PCR directed from total U937 cDNA produced a fragment of DNA encoding several peptides of the 75 kD protein. The PCR synthesized DNA was cloned and used to screen a lambda library. Analysis of the recombinant protein is in progress.

Identification of a 138,000 MW protein which displays a phosphorylation-dependent binding to intron 1 of the c-myc gene

In collaboration with Dr. Maria Zajac-Kaye of the Medicine Branch, NCI, we have identified, substantially purified and characterized a 138,000 MW polypeptide which binds specifically to the site previously shown to be frequently mutated in Burkitt's lymphoma. This protein was shown to be phosphorylated; importantly, specific binding is markedly reduced by dephosphorylation. The modulation of DNA binding by phosphorylation state is a direct effect on the 138,000 MW protein, because the binding activity of the renatured 138,000 MW protein immobilized to nitrocellulose after electroblotting (Southwestern blot) was also phosphorylation dependent.

Recent biochemical studies have demonstrated the presence of a second protein component which is present in the same complex as the 138 kD protein. UV-crosslinking studies label a 35 kD protein. Previous studies have demonstrated a 35 kD phosphoprotein co-fractionating with the 138 kD protein. *In vitro* binding studies have shown complementation between fractions containing these proteins. Functional analysis of the cis element, to which this protein binds, is in progress.

A region from - 100 to -140 bp upstream of c-myc promoter P1 has been demonstrated to bind at least 5 factors. Deletion of this region is associated with a decrease of c-myc transcription, suggesting a positive role for these elements in the regulation of this proto-oncogene. We have purified several of these proteins which possess novel properties. Several of these proteins bind in a sequence specific manner to each of the separate single strands comprising this element. Preliminary *in vivo* and *in vitro* studies indicate that the activity of these factors is mediated through single stranded cis elements.

Major Findings:

1. A 138 kd protein binds in a phosphorylation dependent manner to a cis-element frequently mutated in Burkitt lymphoma. Binding is enhanced by a 35 kD protein.
2. A positive cis-element at -1500 relative to P1 binds a cell type and differentiation specific protein of 75,000 MW.
3. Multiple proteins bind a positive element from -100 to -140 relative to P1. These proteins exert a stimulatory influence on c-myc expression through single-stranded cis elements.

Publications:

Avigan MI, Strober B, Levens D. A far upstream element stimulates c-myc expression in undifferentiated leukemia cells. *J Biol Chem* 1990;265:18538-45.

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

PROJECT NUMBER

Z01 CB 09168-04 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	A. Farina	Visiting Fellow	LP NCI
	K. Gardner	Biotechnology Fellow	LP NCI
	T. Davis	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:

4

PROFESSIONAL:

3.0

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 gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly 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 has a molecular weight of 38-42,000 and is found most abundantly in T cells. The second component is smaller and ubiquitous. A variety of transfection and DNA-binding studies suggests that regulation of the interaction of components one and two may be an important control point for regulating T-cell activation.

Biochemical characterization of the MLA 144 AP1 site binding activity

Ion exchange chromatography of MLA 144 gibbon T-cell nuclear extracts followed by several cycles of oligonucleotide affinity chromatography yield a factor which binds weakly and with reduced specificity to the GALV AP1 site. Seemingly paradoxically, the specific binding of this factor is greatly augmented by a non-DNA binding activity present in the flowthrough of the affinity column. The activity of the retained fraction, termed core, is associated with polypeptides of 38,000 and 42,000 MW. HPLC separation of tryptic peptides revealed these polypeptides to be highly related and most likely to be either processed or modified forms of the same gene product or the products of very highly related genes. The binding and immunologic properties of these proteins suggest that they are not any of the known members of the AP1 family. Although one of three sequenced tryptic peptides of the 42,000 MW protein shows homology to the amino terminal region of both jun-b and jun-d, it is clearly not the gibbon homolog of either of these. This same peptide is present in the 38,000 MW protein providing direct sequence confirmation of the relatedness of the 38,000 and 42,000 MW polypeptides. A variety of binding studies indicate that multimeric participation of the core is required for specific binding to the GALV-AP1 site, most probably as a dimer. The dimerization of core is a reversible mass action-mediated process. Such dimerization has many potential regulatory ramifications.

The flowthrough factor is a 25 kD protein of the GALV-AP1 site binding complex. Binding studies indicate that it participates stoichiometrically in the interaction with the GALV-AP1 site. The interaction of core and flowthrough is reversible and requires no added energy source. The addition of core and flowthrough to nuclear extracts of EL4 (which alone does not support AP1 site mediated transcription) reconstitutes active AP1-site driven transcription.

The dynamics governing the assembly of the GALV-AP1 site complex indicate potential regulatory participation in some physiological process. Preliminary results suggest that this process may include T-cell activation. In response to a variety of stimuli, resting T cells induce a variety of gene products required both for T-cell function and for T-cell proliferation. Many of these induced genes respond to stimulation even in the presence of cycloheximide, strongly implying that their regulatory proteins pre-exist within the T cell prior to activation. Resting Jurkat human T cells have virtually undetectable levels of fos/jun and display very little binding activity for the GALV-AP1 site. Activation of Jurkat cells, even in the presence of cycloheximide or anisomycin, reveals a dramatic increase in AP1 site binding activity. These results suggest induction of an AP1-like factor from components pre-existing in Jurkat cells prior to activation. Importantly, extracts from non-stimulated Jurkat cells display marked biochemical complementation upon the addition of MLA 144 core, which indicates that the flowthrough component of the MLA 144 complex is resident in Jurkat cells and in an active state.

The major GALV-AP1 site binding activity in extracts of Jurkat cells stimulated in the presence of cycloheximide, can be resolved into two components termed core-J and flowthrough-J, which complement each other and which cross-complement the appropriate MLA 144 fractions. Thus it appears that in Jurkat cells, the induction of a major AP1 site binding complex composed of core and flowthrough may be an early event in T-cell activation. In MLA 144 cells, this same complex is constitutively active. Consistent with this notion, the GALV enhancer is very active in MLA 144 cells and further stimulation by TPA does not occur; in contrast, GALV enhancer activity is strongly induced by TPA in Jurkat cells. Additional biochemical and physiological characterization of the core and flowthrough as well as examination of their role in T-cell activation are in progress. Protein-synthesis independent activation of core and flowthrough occurs concomitantly with protein-synthesis independent stimulation of transcription through the GALV-AP1 site *in vivo*.

Major Findings:

1. A core complex composed of a functionally equivalent 38 and 42 kD polypeptides (approximately 35 kD) interacts with a 25 kD factor to produce a specific GALV/AP1 site binding complex. This complex is distinct from fos/jun.
2. A minor complex contains fos-related antigens.
3. The components of the major complex can be activated and regulated in a cell line-specific manner.
4. These components stimulate transcription from the GALV AP1 site *in vitro*.
5. Protein-synthesis independent activation of core and flowthrough occurs concomitantly with protein-synthesis independent stimulation of transcription through the GALV-AP1 site *in vivo*.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09170-04 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Senior Staff Fellow	LP NCI
OTHER:	M. Ranson	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 (research collaborators)

LAB/BRANCH

Laboratory of Pathology

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

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 selectively expressed in limb buds during early development. One of these genes is expressed in a graded fashion along the A-P axis of the limb and is more abundant in wing than leg buds, suggesting possible roles in pattern formation and/or the determination of limb-type identity. The second gene appears to be selectively expressed later in developing limb buds and might play a role in later morphogenetic events such as remodeling. Studies are underway to elucidate the function of these regulatory genes, using both genetic and biochemical approaches.

Major Findings:

Dual approaches are being employed to isolate genes involved in morphogenesis of the chick embryo limb bud. cDNA cloning and subtractive hybridization techniques are being used to identify genes that are differentially expressed at different stages of limb bud development and/or show specificity of expression with regard to limb type. A second, more directed approach will entail the use of cloned genes which have been implicated in developmental regulatory processes (especially pattern formation) in other systems as probes to screen chick limb bud cDNA libraries for related sequences. Genes that are identified using these approaches will be initially analyzed for their specific spatial and temporal patterns of expression in the embryo by use of *in situ* hybridization. These studies may provide insight into factors involved in initiating or maintaining morphogenetic gradients, and clues as to possible hierarchies of interaction/interplay between different factors involved in regulatory circuits during pattern formation. The introduction of cloned genes of interest into defective avian retroviral vectors will provide a means for studying the effects of transient expression in specific tissues by localized injections into developing embryos, as well as potentially allowing for the development of transgenic animals. The isolation and characterization of corresponding genomic clones will also eventually provide the tools necessary to study the transcriptional and post-transcriptional regulation of genes determining pattern formation and of the "down stream" structural genes which they regulate to bring about the developmental program.

For the purpose of generating cDNA libraries and hybridization-subtraction probes, early stage (17-18) and late stage (21-22) limb bud mRNAs were extracted from about 2000 individually dissected chick embryos. These stages were chosen as likely to represent times at which signals regulating anterior-posterior and dorso-ventral patterning are just beginning to be expressed ("early"), and times at which morphogenetic gradients are well established ("late"). The isolation of differentially expressed mRNAs from these different stages should identify genes that are involved in establishing pattern formation, or at the very least, "marker" genes that are regulated by such morphogenetic signals. In addition, considering the profound differences in morphology between fore and hind limb buds in birds which is determined by the mesenchyme of the limb bud at a very early stage, wing and leg bud mRNA populations were extracted separately at the indicated stages, to allow identification of genes differentially expressed in wing or leg limb buds, that may regulate the determination of limb identity.

Because of the relatively small amounts of mRNA that can be isolated even from a large number of limb buds, the different mRNA populations were stably amplified via directional cDNA cloning into a λ bacteriophage vector (λ zap) in which the oriented cDNA insert is flanked by T3 and T7 bacteriophage promoters. The amplified cDNA population can then be transcribed to specifically yield large amounts of either sense or antisense RNA for use in the generation of hybridization subtraction probes. In addition, a modified polymerase chain reaction (pcr) procedure using primers that flank the phage

cdNA inserts is being developed, which would allow the generation of amplified single-stranded cdNA pools for use in subtractive hybridizations. This latter technique could be useful for selectively amplifying the single-stranded (differentially expressed) cdNA isolated after multiple rounds of subtractive hybridization to increase the yield of low abundance sequences.

To date, four different cdNA libraries have been constructed in λ zap using mRNA isolated from early (st 17-18) and late (st 21-22) stage wing and leg buds. Each library contains approximately 10^7 independent clones, ranging in size from 300 to 2600 bp. Modified procedures (as described above) are currently being developed and optimized for enhancing the isolation of differentially expressed, low abundance sequences from these libraries by subtractive hybridization techniques.

Concomitantly, the libraries are also being screened for expression of related/new members of gene families which have been implicated in developmental regulation and pattern formation in other systems. The use of degenerate oligonucleotide primers for pcr amplification of gene segments is particularly useful in identifying new members of highly conserved large multigene families such as homeobox and pou-homeobox genes. Using this strategy, we have found that 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 in limb buds during early development. One of these genes (*Ghox 4.7*) is expressed at 4 to 5 fold higher levels in wing than leg buds and *in situ* hybridizations reveal a graded pattern of expression across the A-P axis of the limb bud, with high levels of expression posteriorly and trace levels of expression as the anterior border is reached. This expression pattern is qualitatively similar to the expression patterns of the morphogen retinoic acid in the limb bud which is known to play a critical role in patterning along the A-P axis. Hence, *Ghox 4.7* may also play a role in determining pattern and/or establishing limb-type identity. A second new homeobox gene (LA-5) is selectively expressed in later stage limb buds and thus may play some role in later morphogenetic events such as limb remodeling via selective growth/programmed death. *In situ* hybridizations are currently underway to determine the spatial pattern of expression of this gene in the developing limb buds. Future experiments to evaluate the role that these genes play during limb development will include determining how their expression is modulated in the context of microsurgical manipulations and by the heterotopic application of morphogens such as retinoic acid.

More long-term experiments to determine the function of these genes will 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 where the effects of transient overexpression or ablation of expression can be evaluated by localized injections of virus into selected tissues of developing embryos at different stages during development. These sorts of genetic analyses are

already underway 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 partial and complete coding sequence constructs have been made for introduction as transgenes into mice. For the production of transgenic mice, a promoter which gives high level expression primarily in the limb buds of developing embryos (a truncated *Hox 2.2* promoter) has been used to drive expression in order to target (and restrict) overexpression of *Ghox 4.7* to the developing limb bud to prevent the possible occurrence of early embryonic lethals due to toxicity of ubiquitous expression.

Biochemical approaches are also being employed to identify downstream "targets" that are regulated by *Ghox 4.7* during limb morphogenesis. This work will begin with identification of the cis DNA sequence elements to which the *Ghox 4.7* protein binds. Specific antibodies against the *Ghox 4.7* protein have been raised, and these will be useful in identification and characterization of cis elements that *Ghox 4.7* can bind to *in vitro*, and eventually useful in the isolation of *in vivo* complexes formed between the *Ghox 4.7* protein and DNA in limb buds 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: (in press)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09171-08 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	P. Rohan	Biotechnology Fellow	LP NCI
	K. Smith	Breast Cancer Fellow	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:

1.15

PROFESSIONAL:

0.4

OTHER:

0.75

CHECK APPROPRIATE BOX(ES)

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

B

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

We are 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 a large number of early induced genes code for transcription factors. A variety of structural classes of factors have been observed, including 3 zinc-finger containing proteins (pAT 225, 133, and 591), a protein related to the steroid receptor family of transcription factors (pAT 416), and two *rel*-related members of the NF- κ B-binding transcription complex (pAT 243 and 189). Additional clones have been described for a putative GTP-binding protein (pAT 270, RAI-1), a phosphotyrosine phosphatase (pAT 120, CAP-1), a hematopoietic cell-specific membrane protein (pAT 237), and a 158 amino acid peptide that contains a hydrophobic leader sequence, but appears to be cell-associated (pAT 154). Structural and functional analyses are proceeding on selected clones.

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.

Primary sequence analysis on two clones, pAT 243 and pAT 189, revealed that the proteins encoded by these cDNA's are rel-related family members of the NF-KB transcription factor complex. Therefore, since the detailed study of these genes and the proteins encoded by them encompasses a variety of questions and a large body of work, this is now being considered as a new project (Z01 CB 09356-01 LP). Similarly, primary sequence analysis of pAT 270 has shown that the encoded protein is a member of the ras-related family of proteins. The characterization of the structure and function of the pAT 270 protein (renamed RAI-1) includes a number of experimental approaches and is being considered as a new project (Z01 CB 09357-01 LP). Comparison of the predicted protein encoded by pAT 120 to current protein data bases utilizing the "blast" program has revealed a conserved consensus sequence found in phosphotyrosine phosphatases. The characterization of the structure and biological function of the pAT 120 protein (renamed CAP-1) is presented as a new project (Z01 CB 09358-01 LP).

pAT 237 appears to encode a hematopoietic cell-specific, mitogen inducible type II membrane protein. The potential role of the 237 protein as a receptor for soluble or cell-bound ligand is being investigated. The approach we have taken is to attempt to mimic ligand binding by using monoclonal antibodies directed to the external region of the 237 protein. To this end, we have produced a recombinant protein composed of the T7 phage 10 protein joined to the carboxy-terminal third of the 237 protein. The recombinant protein has been used to elicit polyclonal rabbit antibodies and is currently being used to elicit murine monoclonal antibodies. Monoclonal antibodies will be screened using ELISA, Western blotting, and immunoprecipitation. Appropriate antibodies will be tested for stimulating or blocking cell-mediated immune reactions.

pAT 154 encodes a 158 amino acid peptide with a putative hydrophobic leader sequence. pAT message is seen predominantly in hematopoietic tissues and lung. Polyclonal rabbit antisera have been generated against recombinant 154 protein. These antisera precipitate a cell-associated protein of approximately 16 kd from COS cells transfected with a 154 expression vector construct and from mitogen-stimulated T cells. No protein is observed in the culture supernatants which suggests that 154 protein may be associated with a membrane structure through its hydrophobic leader sequence. Therefore, immunohistochemistry is currently in progress to establish the subcellular location of the 154 protein.

Additional characterization of mitogen-induced clones has revealed that pAT 591 and pAT 133 encode zinc-finger-containing proteins that respectively are homologous to a previously described murine protein, erg-2, and have been

previously undescribed. pAT 281 and pAT 563 encode open reading frames with no obvious structural features or homologies to suggest function at this time. Sequence determinations for full-length pAT 276 clones are in progress.

Publications:

Irving SG, Zipfel PF, Balke J, McBride OW, Morton CC, Burd PR, Siebenlist U, Kelly K. Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q. Nucl Acids Res 1990;18:3261-70.

Bours V, Villalobos J, Burd PR, Kelly K, Siebenlist U. Cloning of a mitogen-inducible gene encoding a kB DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature 1990;348:76-80.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09356-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structural and Functional Characterization of NF-KB Binding Transcription Factors

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

PI:	K. Kelly	Senior Staff Fellow	LP NCI
	U. Siebenlist	Senior Staff Fellow	LIR NIAID
OTHER:	K. Smith	Breast Cancer Fellow	LP NCI

COOPERATING UNITS (if any)

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

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

B

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

We have cloned and characterized two mitogen-inducible genes from human peripheral blood T cells that comprise members of the NF-kB family of transcription factors. Biochemical studies previously have defined NF-kB as a heterodimer of 50 kD (p50) and 65 kD (p65). We have shown that pAT 243 encodes a 105 kD precursor protein for p50 that becomes processed, resulting in an amino terminal 50 kD protein that shows binding activity to an NF-kB binding site, either as a homodimer or a heterodimer with p65. The amino-terminal domain has regions homologous to the oncogene rel. The carboxy-terminal domain contains repeat structures that are found in a variety of proteins and that probably determine protein-protein interactions. We have characterized an additional gene, pAT 189, that resembles the structural organization of p105 and shares 44% amino acid homology with it. Truncated 189 protein binds an NF-kB consensus sequence as a homodimer or as a heterodimer with p50 or p65. Thus, it appears that NF-kB transcription factors are unexpectedly composed of a family of polypeptides that at a minimum include p50, pAT 189-encoded protein, c-rel, and p65.

Major Findings:

We have cloned and characterized a mitogen-inducible gene, pAT 243, isolated from human T cells that predicts a protein of 968 amino acids. The amino-terminal domain has regions homologous to the oncogene *rel* and to the developmentally important gene *dorsal* of *Drosophila*. The carboxy-terminal domain contains repeat structures found in a variety of proteins that are involved in cell-cycle control of yeast and in tissue differentiation in *Drosophila* and *Caenorhabditis elegans*, as well as in the putative human oncogene *bcl-3* and in the ankyrin protein. A truncated form of the product of this gene translated *in vitro* is a DNA-binding protein which interacts specifically with the KB binding site found in many inducible genes, including the enhancer in human immunodeficiency virus. A comparison to the sequence of genes encoding the mouse and human p50 subunit of NF-KB show that pAT 243 is identical to the human p50-encoding gene. The nonprocessed form of p50 has been designated p105.

An additional mitogen-inducible gene, pAT 189, has been characterized and found to encode a protein that resembles the structural organization of p105. Although there is no DNA cross-hybridization between pAT 243 and 189, the encoded proteins share 44% amino acid homology overall and 61% amino acid homology in the *rel*-related region. *In vitro* translated, truncated 189 protein binds an NF-kB consensus sequence as a homodimer or as a heterodimer with p50 or other *rel*-related proteins that do not require post-translational processing (p65, c-rel, and rel-B). Thus, the NF-KB family of transcription factors appears to be composed of a variety of polypeptides that can combine in various pairs to bind NF-KB sites *in vitro*. Future and current efforts are directed at: 1) establishing structure/function relationships that may give clues to the mechanism of v-rel transformation, 2) determining the regulation of NF-KB complex family members at the transcriptional and post-translational level, and 3) investigating the role of various NF-KB complexes in the regulation of HIV and other induced gene expression.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09357-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any)

Ulrich Siebenlist, Ph.D., Laboratory of Immune Regulation, NIAID, NIH

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Laboratory of Pathology

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Office of the Chief

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.3

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

B

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

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 32 kdalton protein that contains consensus GTP binding elements, although the G-3 element is imperfect. 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 T cells and fibroblasts and in PMA-activated blood monocytes. The specific biological function of RAI-1 is not known as yet, although it should be noted that low molecular weight GTP binding proteins act as molecular switches in the cell for functions as diverse as receptor signalling and vesicle transport. The GTP binding activity of native RAI-1 protein and the subcellular location of RAI-1 are being investigated.

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, pAT 270, encodes a 32 kd protein (confirmed by *in vitro* transcription and translation) 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 consensus elements, although the G-3 region is imperfect. RAI-1 demonstrates amino and carboxy terminal extensions relative to ras-related proteins. In addition, there is no consensus isoprenylation site at the carboxy terminus of RAI-1, although there are cysteines near the carboxy terminus that could serve as fatty acid addition sites. RAI-1 maps to the long arm of human chromosome 8.

RAI-1 mRNA is transiently expressed in T cells and fibroblasts between 2 and 8 hours after mitogen activation. This is the first description of a ras-related protein that is substantially transcriptionally-regulated. Preliminary experiments suggest that constitutive RAI-1 expression is non-transforming in an NIH 3T3 cell assay and non-reverting for v-ras transformed NIH 3T3 cells.

Current efforts are directed at establishing the biological role of RAI-1. First, does RAI-1 bind GTP and act as a GTPase? Recombinant RAI-1 fusion proteins are insoluble. Solubilized and partially renatured RAI-1 protein does not bind GTP. However, because large proteins often do not renature sufficiently to regain substrate-binding or enzymatic activity, we are presently developing an immunoaffinity purification scheme to allow isolation of native RAI-1 protein. Various anti-peptide and anti-protein antibody reagents are being tested. These reagents also will be utilized to establish the subcellular localization of RAI-1. This information should assist in establishing a biological role for RAI-1.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09358-01 LP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any)

Ulrich Siebenlist, Ph.D., Laboratory of Immune Regulation, NIAID, NIH

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Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.1

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

B

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

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 consensus 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 T cells, B cells, and fibroblasts. Consistent with expression in activated or cycling cells, CAP-1 message is also found in spleen, thymus, and bone marrow, but not in a variety of other tissues such as liver, kidney, heart, and muscle. Transfection of CAP-1 expression vectors into NIH 3T3 cells has suggested that constitutive CAP-1 expression prevents clonal selection, and therefore may be inhibitory for growth or a so-called tumor suppressor gene.

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 consensus 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, although a potential myristylation site exists, suggesting that CAP-1 protein may be membrane-associated. 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. The CAP-1 locus has been mapped to the short arm of human chromosome 2.

CAP-1 mRNA is transiently expressed between 1 and 3 hours following mitogen activation of T cells, B cells, and fibroblasts. CAP-1 is unique in this expression property relative to other described PTPases that are constitutively expressed. CAP-1 mRNA is observed in murine or human spleen, thymus, and bone marrow but not in murine heart, lung, liver, kidney, stomach, or muscle. Such a profile is consistent with the expression of CAP-1 in activated lymphoid cells. In addition, CAP-1 message is constitutively expressed in HTLV-1 infected, human T cell clones. The basis of this expression appears to be transcriptional activation by the HTLV-1 tax gene product. Structural analysis of the CAP-1 promoter is planned to determine which element(s) within the upstream regulatory region contribute to such deregulated expression. Toward this end, genomic clones containing the CAP-1 gene and adjacent 5' DNA have been isolated and partially characterized.

In order to characterize CAP-1 protein, polyclonal rabbit anti-peptide antibodies to the predicted protein sequence have been generated. Such an antibody to an amino-terminal peptide immunoprecipitates radiolabeled protein of the expected size from COS cells transfected with eukaryotic expression vector constructs containing CAP-1 cDNA. Polyclonal rabbit antibodies also have been raised against a recombinant hybrid protein consisting of the phage T7 gene 10 product fused to the carboxy terminal half of CAP-1. The complete CAP-1 protein is unstable in *E. coli* and therefore cannot be produced. Such antibodies will be utilized to further characterize CAP-1 protein with regard to post-translational modifications and subcellular localization, and to immunoprecipitate enzymatically active protein in order to confirm the predicted tyrosine phosphatase activity of CAP-1.

The biological function of CAP-1 is an important question. Phosphotyrosine phosphatases have been predicted to be growth suppressor genes because they counterbalance the activity of the growth-transforming tyrosine kinases. In support of this idea, we have found in preliminary experiments that constitutive CAP-1 expression inhibits the growth of murine NIH 3T3 cells. The effect of constitutive and inducible CAP-1 expression on additional cell

types, including some transformed with tyrosine kinase oncogenes, is currently being tested.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBDC, NCI

October 1, 1990 through September 30, 1991

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. In addition we are assessing changes in epidermis-derived cytokine mRNA levels early in the afferent phase of contact sensitivity. We are using a sensitive reverse transcriptase-PCR-technique to quantitatively compare the patterns of mRNA regulation of the following: class II MHC I-A α , TNF- α , IL-1 α , IL-1 β , interferon (IFN)- γ , GM-CSF, IFN-induced protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2). Enhanced LC-derived IL-1 β 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-A α , IL-1 α , IL-1 β , 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-1 β and class II MHC I-A α mRNA's, keratinocytes are the primary source of the TNF- α , IL-1 α , IP-10 and MIP-2, and infiltrating T lymphocytes are the source of the IFN- γ . Relevance of the molecular findings is being sought by attempting to identify biologically active IL-1 α and immunoreactive TNF- α and IFN- γ in culture supernatants. The studies, to date, demonstrate that LC-derived and certain keratinocyte-derived cytokine mRNAs are selectively upregulated by allergens in the very early afferent phase of contact sensitivity. We are now technically able to assess the role of physical agents (i.e. UV) as well as other chemical agents and even infectious agents (i.e. H. Simplex and HIV) to affect epidermis-derived cytokines and thereby affect inflammatory and immune responses in the skin.

Regulation of Cutaneous Accessory Cell Activity in Health and Disease
(Dr. Mark Udey):

A major focus of this laboratory is to determine precisely how UV radiation inhibits the accessory cell function of epidermal Langerhans cells (LC). We have found that exposure of epidermal cells (EC) to low doses of UV-B radiation ($\leq 200 \text{ J/m}^2$) in vitro completely inhibited the ability of freshly-isolated LC (fLC) to support T cell proliferation in an anti-CD3 induced mitogenic T cell assay. This inhibition resulted from a direct effect of UV-B on LC and was not mediated by irradiated keratinocytes (KC) or their products. The effects of UV-B on LC accessory cell function also was not reversed by exogenous GM-CSF or TNF α (two KC-derived cytokines which promote LC survival in vitro), or the T cell costimulatory cytokines IL-1 and IL-6.

To begin to define the photoreceptor(s) responsible for the inhibitory activities of UV-B radiation and elucidate the mechanism(s) by which these activities are expressed, we examined the effect of several other types of UV (other than UV-B) radiation on LC accessory cell function, ICAM-1 expression and LC survival in vitro. UV-C radiation inhibited ICAM-1 expression and LC accessory cell activity, albeit at lower doses than UV-B radiation (with essentially complete inhibition occurring at 20 J/m^2). UV-A radiation (0.25 J/cm^2) inhibited LC ICAM-1 expression and function only if cells had been pre-treated with 8-methoxypsoralen. Careful examination of the effects of UV-C and psoralen + UV-A (PUVA) radiation on the survival of fLC in vitro revealed that although similar numbers of LC were recovered from cultures seeded 24 h earlier with unirradiated LC or LC that had been exposed to UV-C or PUVA, recovery of irradiated LC at 48 and 72 h was only 10% of that from cultures of unirradiated EC.

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. Bullous pemphigoid (BP) is known to be a component of the hemidesmosome, a basal cell-substrate adhesion junction. We have determined, with immunochemical methods, that the BP antigen is a 230 kD protein with a pI of 8. We have begun using the PCR for the rapid amplification of cDNA ends (RACE) to finish cloning the 5' end of the BP cDNA. In addition, we have begun characterization of the BP antigen gene in normal humans, animals, and patients with junctional epidermolysis bullosa, a disease with abnormal hemidesmosomes. With immunochemical methods we have defined a new form of pemphigus, paraneoplastic pemphigus, that is clinically, histologically, and molecularly unique and is associated with lymphoma. Finally, we have started cDNA cloning of the pemphigus vulgaris (another antibody-mediated disease) antigen by antibody screening of a λ gt11 expression cDNA library derived from cultured keratinocytes. We are raising antibodies against fusion proteins derived from this cDNA. These should prove useful for studying the localization of the pemphigus vulgaris antigen as well as the pathophysiology of this disease.

Therapy of Skin Cancer and Disorders of Keratinization (Dr. John DiGiovanna):

The goal of our 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. During the last decade over 300 patients have been enrolled in protocols which have established the efficacy and characterized the toxicity of isotretinoin and etretinate in the treatment of a variety of dermatologic disorders. 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.

We are expanding our studies directed at the treatment and chemoprevention of skin cancer. The efficacy and toxicity of isotretinoin as a chemopreventive agent is being further studied in a series of 8 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 probably occurring at a late stage of carcinogenesis. Statistically, the beneficial effect was highly significant. After one year without isotretinoin, patients were started 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 effect. Currently, those patients with an inadequate response to low dose (0.5 mg/kg/day) are being treated at an intermediate dose (1.0 mg/kg/day) in an attempt to determine an adequate chemopreventing dose with minimum toxicity.

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. 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, retinitis pigmentosa, and Friedreich ataxia. These studies are designed to elucidate the pathogenesis of these disorders. In collaboration with Dr. Katherine S. Sanford and Dr. Ram Parshad (Department of Pathology, Howard University College of Medicine) we are studying DNA repair in cells from patients with Alzheimer disease (AD). We are attempting to develop a cytogenetic test which can distinguish AD from normal cells. Exposure of human cells in culture to fluorescent light produces several DNA lesions, including strand breaks and base damage. The breaks, together with indirect breaks formed during repair of base damage, can

be quantified cytogenetically as chromatid aberrations (i.e., chromatid breaks and gaps) at the subsequent metaphase. We found that when exposed during the G₁-phase skin fibroblasts from patients with AD (2 familial and 2 sporadic cases) had frequencies of chromatid aberrations similar to those of fibroblasts from 4 age-matched normal donors. However, addition of the DNA-repair inhibitor caffeine during the S-phase following G₁-phase light exposure significantly increased frequencies of chromatid aberrations only in the AD cells. These results suggest that damage repaired in normal cells during the G₁-phase persists and is repaired in the AD cells during S-phase by a caffeine-sensitive repair mechanism. Addition of cytosine arabinoside (an inhibitor of DNA repair synthesis) to cells after G₂-phase light exposure significantly increased chromatid aberrations in the 2 normal, but not the 2 AD lines tested, indicating that normal but not AD cells have incision activity for removal of base damage during the G₂-phase. We infer from our results that lesions persist in AD cells during the G₁- and G₂-phases but are repaired during the S-phase. The abnormal cytogenetic responses of the AD cells may provide the basis for a test to predict which persons at risk for AD will develop the disease and suggest that the premature death of postmitotic neurons in AD may be caused by accumulation of unrepaired DNA lesions.

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

PROJECT NUMBER
Z01 CB 03638-22 D

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
A. J. Fornace, Jr., M.D., Senior Investigator, LMPH, DCT, NCI
K. H. Kraemer, M.D., Senior Investigator, LMC, DCE, 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 Molecular Carcinogenesis, DCE, NCI; Laboratory of Cellular and Molecular Biology, DCE, NCI; Biostatistics Branch, DCCP, NCI

LAB/BRANCH

Dermatology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.8

PROFESSIONAL:

1.3

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

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

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, retinitis pigmentosa, and Friedreich ataxia. These studies are designed to elucidate the pathogenesis of these disorders. We assess the biological effectiveness of DNA repair by: 1) in vitro assays of cell survival after treatment of the cells with DNA-damaging agents; 2) analysis of chromosomal and chromatid aberrations in cells treated with DNA-damaging agents; and 3) transfection studies using irradiated plasmids. We study DNA repair in the overall genome of cultured cells and within active genes. We search in patient cells for abnormalities in DNA-repair genes and in their DNA damage-inducible transcripts using cDNA probes in Southern and Northern blot hybridization studies.

Project DescriptionMajor Findings:

In collaboration with Dr. Katherine S. Sanford and Dr. Ram Parshad (Department of Pathology, Howard University College of Medicine) we are studying DNA repair in cells from patients with Alzheimer disease (AD). We are attempting to develop a cytogenetic test which can distinguish AD from normal cells. Exposure of human cells in culture to fluorescent light produces several DNA lesions, including strand breaks and base damage. The breaks, together with indirect breaks formed during repair of base damage, can be quantified cytogenetically as chromatid aberrations (i.e., chromatid breaks and gaps) at the subsequent metaphase. We found that when exposed during the G₁-phase skin fibroblasts from patients with AD (2 familial and 2 sporadic cases) had frequencies of chromatid aberrations similar to those of fibroblasts from 4 age-matched normal donors. However, addition of the DNA-repair inhibitor caffeine during the S-phase following G₁-phase light exposure significantly increased frequencies of chromatid aberrations only in the AD cells. These results suggest that damage repaired in normal cells during the G₁-phase persists and is repaired in the AD cells during S-phase by a caffeine-sensitive repair mechanism. Addition of cytosine arabinoside (an inhibitor of DNA repair synthesis) to cells after G₂-phase light exposure significantly increased chromatid aberrations in the 2 normal, but not the 2 AD lines tested, indicating that normal but not AD cells have incision activity for removal of base damage during the G₂-phase. We infer from our results that lesions persist in AD cells during the G₁- and G₂-phases but are repaired during the S-phase. The abnormal cytogenetic responses of the AD cells may provide the basis for a test to predict which persons at risk for AD will develop the disease and suggest that the premature death of postmitotic neurons in AD may be caused by accumulation of unrepaired DNA lesions.

In collaboration with Drs. Vilhelm A. Bohr, Michele K. Evans and Charles Link, we are studying the repair of ultraviolet radiation (UV)-induced cyclobutane pyrimidine dimers in an active housekeeping gene [the dihydrofolate reductase (DHFR) gene] as opposed to the conventional study of repair in the genome overall. In this procedure isolated DNA from fibroblasts is restricted, separated from replicated DNA, nicked at dimers with T-4 endonuclease V, electrophoresed in alkaline agarose, and subjected to Southern hybridization. We have studied normal and XP group A, C, and F fibroblast strains. We have found all the XP strains to have defective removal of dimers from the DHFR gene. The cells' relative capacity to repair the gene is the same as their relative capacity to perform UV-induced unscheduled DNA synthesis in the genome overall and does not correlate with the cells' relative ability to survive the lethal effects of UV. These studies show that removal of dimers from an active gene is not required for high survival, since the relatively UV-resistant group F strain showed virtually no dimer removal. Our results indicate that some lesions(s) other than dimers is likely to be important in UV lethality. We plan to adapt the active-gene-repair assay to monitor the repair of nondimer lesions induced by UV, for example, the 6-4 photoproduct. We are studying active-gene-repair in Cockayne syndrome cells in an attempt to

confirm reports of others that in Cockayne syndrome there is defective DNA repair in active genes but not in the genome overall. We are studying also the repair of alkylation damage in active genes of patients with Alzheimer disease.

All XP patients have abnormally increased frequencies of UV-induced skin cancers. It is known that nucleotide excision-repair-deficient XP patients have abnormally high levels of UV-induced chromosomal aberrations. We are continuing our studies to determine the levels of such aberrations in cells from XP variant patients (who have normal levels of nucleotide excision repair). These studies may indicate whether or not abnormal levels of UV-induced chromosomal aberrations are causally related to the development of sunlight-induced skin cancer in XP patients. Our results have shown that XP variant cells do not have a significantly higher level of UV-induced chromosomal aberrations than normal cells. We are now attempting to determine whether caffeine will increase the number of UV-induced chromosomal aberrations in the XP variant cells above the number in normal cells.

We had previously reported that a patient with both XP and Cockayne syndrome (the XP-CS complex) comprised XP complementation group H, since we believed his cells complemented reference strains from all known XP complementation groups, including group D. However, scientists in The Netherlands and in Italy were unable to obtain complementation between the group-H strain and group-D strains, although scientists in Japan have reported such complementation. We have performed new complementation studies which show that the patient we considered to comprise group H is actually in group D. In reviewing our original study, we now conclude that cells in S-phase DNA synthesis were mistakenly considered to be undergoing unscheduled DNA synthesis and to represent complementing cells. We have, therefore withdrawn our claim of the existence of group H and have reassigned the patient to group D.

XP neurological disease has previously been considered to be a disease only of children, since all reported cases have had symptomatic onset prior to 21 years of age. The age of onset has been shown to be closely related to the severity of the patients' DNA-repair defects, as measured by the survival of the patients' cultured skin fibroblasts after their exposure to UV radiation in vitro. We have now investigated whether or not XP neurological disease occurs in adult XP patients, and we have found a 44-year-old group-C patient to have a progressive form of asymptomatic XP neurological disease. The survival of her irradiated cells is in the range given by cells from patients with the late-onset type of the juvenile-onset form of XP neurological disease. We are also studying cells from an unusual group-A patient from Egypt (who had no neurological abnormalities by age 35 years). Colony-forming ability studies of his cells after UV-irradiation are being conducted, and the results will be compared to those from other group-A patients.

We have completed our collaboration with Dr. Kenneth H. Kraemer in which we transiently transfected human lymphoblastoid cell lines with a plasmid (pRSVcat) containing the bacterial cat gene coding for chloramphenicol acetyltransferase. The plasmid was irradiated with different doses of 254-nm

UV. Then, in order to evaluate the repair of dimer and nondimer photoproducts, aliquots containing the irradiated plasmid were treated with photoreactivating enzyme (E. coli photolyase) and light, a treatment which monomerizes the UV-induced cyclobutane-type of pyrimidine dimer and leaves only nondimer photoproducts. We found that Cockayne syndrome cells are unable to repair pyrimidine dimers induced in the plasmid by UV but can repair normally the nondimer photoproducts, while XP cells are unable to repair either dimer or nondimer photoproducts. These results suggest explanations for the fact that XP patients, but not Cockayne syndrome patients, develop skin cancers.

We have completed our collaboration with Dr. Albert J. Fornace, Jr., on the expression of 2 transcripts which are induced in normal human cells and in radiosensitive ataxia telangiectasia cells by several DNA-damaging agents and by growth arrest. We have found that one of these genes is rapidly induced by x-rays and that its induction is not mediated through protein kinase C. Ataxia telangiectasia cells have defective induction of the gene. This is the first non-protein-kinase-C inducible gene shown to be induced by ionizing radiation and indicates that there exists a previously undiscovered regulatory process related to DNA-damage induced by x-rays.

Publications:

Papathanasiou MA, Kerr NCK, Robbins JH, McBride OW, Alamo Jr I, Barrett SF, Hickson ID, Fornace Jr AJ. Induction by ionizing radiation of the gadd45 gene in cultured human cells: Lack of medication by protein kinase C, Mol Cell Biol 1991;11:1009-1016.

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 (in press).

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 (in press).

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

PROJECT NUMBER
Z01 CB 03657-17 D

PERIOD COVERED
October 1, 1990 to September 30, 1991

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-S. Lee, Visiting Fellow, Dermatology Branch, DCBDC, NCI
E. Dugan, Medical Staff Fellow, Dermatology Branch, DCBDC, NCI
A. Enk, Special Volunteer, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)
Dermatology Dept., USUHS, Bethesda
Immunology Branch, DCBDC, NCI

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	6	PROFESSIONAL:	5	OTHER:	1
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CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard 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. We have, therefore, attempted to utilize this system for the generation of primary in vitro responses to tumor-associated antigens such as the Friend Leukemia Virus associated gp-70. We are also currently assessing the feasibility of performing these studies in cells derived from human beings. 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.

Project DescriptionMajor Findings:

We have found that when murine epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells for 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 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. In addition we are assessing changes in epidermis-derived cytokine mRNA levels early in the afferent phase of contact sensitivity. We are using a sensitive reverse transcriptase-PCR-technique to quantitatively compare the patterns of mRNA regulation of the following: class II MHC I-A α , TNF- α , IL-1 α , IL-1 β , interferon (IFN)- γ , GM-CSF, IFN-induced protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2). Enhanced LC-derived IL-1 β 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-A α , IL-1 α , IL-1 β , 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-1 β and class II MHC I-A α mRNA's, keratinocytes are the primary source of the TNF- α , IL-1 α , IP-10 and MIP-2, and infiltrating T lymphocytes are the source of the IFN- γ . Relevance of the molecular findings is being sought by attempting to identify biologically active IL-1 α and immunoreactive TNF- α and IFN- γ in culture supernatants. The studies, to date, demonstrate that LC-derived and certain keratinocyte-derived cytokine mRNAs are selectively upregulated by allergens in the very early afferent phase of contact sensitivity. (Time devoted to AIDS is 20%).

We are continuing studies of the murine Thy-1⁺ DEC in which we are attempting to identify the function(s) of these cells. The Thy-1⁺ DEC represent an epidermally-located dendritic T cell which has a $\gamma\delta$ T cell receptor. Our studies are utilizing various sources of heat-shock proteins as potential target molecules for these cells.

Publications:

Aiba S, Katz SI. Phenotypic and functional characteristics of in vivo-activated Langerhans cells, J Immunol 1990;145:2791-2796.

Aiba S, Katz SI. The ability of cultured Langerhans cells to process and present protein antigens is MHC-dependent, J Immunol 1991;146:2479-2487.

Shimada S, Caughman SW, Bluestone, JA, Cron RQ, Owen FL, Smith JA, Katz SI. Freshly isolated Thy-1⁺ dendritic epidermal cells express T cell receptor $\gamma\delta$ -CD3, J Dermatol Sci 1990;1:459-464.

Kobata T, Shinkai Y, Iigo Y, Kawasaki A, Yagita H, Ito S, Shimada S, Katz SI, Okumura K. Thy-1⁺ positive dendritic epidermal cells contain a killer protein perforin, Internat'l Immunol 1990;2:1113-1116.

Hauser C, Katz SI. Generation and characterization of T-helper cells by primary in vitro sensitization using Langerhans cells, Immunol Review 1990;117:67-84.

Cooper KD, Androphy EJ, Lowy D, Katz SI. Antigen presentation and T-cell activation in epidermodysplasia verruciformis, J Invest Dermatol 1990;94:769-776.

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

PROJECT NUMBER
 Z01 CB 03659-17 D

PERIOD COVERED
 October 1, 1990 to September 30, 1991

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
 Donita Abangan, Medical Officer, Dermatology Branch, DCBDC, NCI
 T. Peter Bridge, Senior Investigator and Aids Clinical Coordinator,
 Laboratory of Clinical Science, NIMH
 Nicholas Patronas, Radiology Department, Clinical Center

~~XXXXXXXXXXXX~~

Sherrri Bale, Geneticist, Family Studies Section, EEB, DCE
 Peter Steinert, Chief, Lab. Skin Biology, NIAMS

LAB/BRANCH
 Dermatology Branch

SECTION

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.9

PROFESSIONAL: 1.9

OTHER:

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

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

The goal of our 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. During the last decade over 300 patients have been enrolled in protocols which have established the efficacy and characterized the toxicity of isotretinoin and etretinate in the treatment of a variety of dermatologic 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. A clinical trial of intranasal Peptide T for psoriasis was completed and showed minimal efficacy. We are expanding our studies directed at the treatment and chemoprevention of skin cancer. We demonstrated that oral isotretinoin is an effective 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 intralesional recombinant human interferon gamma for basal cell carcinoma is ongoing. All five tumors treated to date have become smaller; one underwent complete histological regression. This study is continuing with higher doses being used to try to improve efficacy. We are beginning a collaborative study to clarify the clinical spectrum and identify the genetic chromosomal localization of a variety of genodermatoses for those conditions where we have suspect candidate genes have been cloned.

Project DescriptionMajor Findings:

The efficacy and toxicity of isotretinoin as a chemopreventive agent is being further studied in a series of 8 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 probably occurring at a late stage of carcinogenesis. Statistically, the beneficial effect was highly significant. All patients had mucocutaneous side effects, many had laboratory abnormalities and two had skeletal toxicity. After one year without isotretinoin, patients were started 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 effect. Currently, those patients with an inadequate response to low dose (0.5 mg/kg/day) are being treated at an intermediate dose (1.0 mg/kg/day) in an attempt to determine an adequate chemopreventing dose with minimum toxicity.

We have documented the efficacy and toxicity of systemic retinoids in a large number of patients over a long period. Patients with a variety of ichthyoses, Darier's disease, pityriasis rubra pilaris, and related keratinizing disorders have maintained clinical improvement for more than a decade while being treated with either isotretinoin and/or etretinate. Most of these conditions have no effective alternative therapy. While treating these patients 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. For retinoids such as etretinate which is fat stored, this hazard can persist after the discontinuation of treatment. Our study of the pharmacokinetics of etretinate elimination measured detectable blood levels 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 elimination of this fat-soluble 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 also 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 peripheral skeletal tissue involvement in etretinate treated patients. We have also observed this peripheral skeletal toxicity after

isotretinoin. Our unique ability for long-term follow-up and monitoring of these patients will enable us to further define these toxicities. Other significant adverse reactions to retinoids have been identified and characterized. Seven patients were observed to develop symptoms of severe depression during oral isotretinoin treatment. Depression completely resolved within 2 to 7 days post treatment. After rechallenge at a lower dose, one patient developed depressive symptoms again. Patients with Darier's disease were noted to have a novel, cystic bone abnormality. Ten of 17 patients surveyed had cystic bone lesions, one had a history of fractures. Bone cysts were formed in patients on retinoid therapy but also in patients who had never been treated with retinoids. These lesions will be further characterized.

Six patients with psoriasis were treated for eight weeks with three dose levels of intranasal Peptide T while following skin changes, neuropsychiatric parameters and pharmacokinetics. Peptide T, which interacts with the CD4 receptor, is being studied 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. The only toxicity noted was nasal stuffiness with sneezing, associated with the intranasal administration. One patient with a history of allergic rhinitis discontinued the study because of an increase in nasal symptoms. Only minimal benefit was observed on the psoriasis using the intranasal preparation. A follow-up study utilizing a topical preparation applied directly to psoriatic lesions is underway. Three patients have been started on this double-blind trial. Time devoted to these AIDS-related studies is 10%.

Five patients have each had one basal cell carcinoma treated with 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 study will be continued for a second phase using a higher dose of gamma interferon to try to achieve greater efficacy.

Publications:

Scheinman PL, Peck GL, Rubinow DR, DiGiovanna JJ, Abangan DL, Ravin PD. Acute Depression from Isotretinoin. J Am Acad Derm 1990;22(6 Pt 1):1112-4.

Milton GP, Peck GL, Fu JL, DiGiovanna JJ, Nordland JJ, Thomas JH, and Sanders SF. Exacerbation of Darier's Disease by lithium carbonate. J Am Acad Derm 1990;23(5 Pt 1):926-8.

Kraemer KH, Seetharam S, Seidman MM, Bredberg A, Brash D, Waters HL, Protic-Sabljić M, Peck G, DiGiovanna J, Moshell A, Tarone RE, Jones G, Parshad R, and Sanford K. Defective DNA repair in humans: Clinical and molecular studies of Xeroderma pigmentosum, in DNA Damage and Repair in Human Tissues, Sutherland BM and Woodhead AD (Eds), Plenum Press, New York, 1990.

Tokar IP, Kraemer KH, and DiGiovanna JJ. Xeroderma pigmentosum: A nursing perspective. Dermatology Nursing 1990;2(6):319-27.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03667-07 D

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecules Defined by Autoantibody - Mediated Skin Diseases

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

P.I.: John R. Stanley, M.D., Medical Officer, Dermatology Branch, DCBDC, NCI

OTHER: Masayuki Amagai, M.D., Visiting Fellow, Dermatology Branch, DCBDC, NCI
George Elgart, M.D., Medical Staff Fellow, Dermatology Br., DCBDC, NCI
Lisa Kauffman, 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:

5

PROFESSIONAL:

4

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

B

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

The general and long-term goal of my laboratory is to study autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone. Specifically, antibodies in these diseases define molecules in the normal epidermis. We are characterizing, at an immunochemical and molecular biologic level, the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). BP antigen is known to be a component of the hemidesmosome, a basal cell-substrate adhesion junction. With extension cDNA cloning, using a previously-isolated partial cDNA, we have cloned overlapping cDNAs encoding almost the total BP antigen molecule. Analysis of protein structure and amino acid sequence indicates marked homology with desmoplakin I, a desmosome plaque protein. These studies indicate that BP antigen and desmoplakin I form a gene family of adhesion plaque proteins. We are now using the polymerase chain reaction (PCR) for the rapid amplification of cDNA ends (RACE) to finish cloning the 5' end of the cDNA. In addition, we have begun characterization of the BP antigen gene in normal humans, animals, and patients with junctional epidermolysis bullosa, a disease with abnormal hemidesmosomes. With immunochemical methods we have defined a new form of pemphigus, paraneoplastic pemphigus, that is clinically, histologically, and molecularly unique. Finally, we have started cDNA cloning of the PV antigen by antibody screening of a λ gt11 expression cDNA library derived from cultured keratinocytes. We are raising antibodies against fusion proteins derived from this cDNA. These should prove useful for studying localization of the PV antigen as well as pathophysiology of disease.

Project Description

Major Findings:

Overlapping cDNAs encoding almost the entire approx. 230 kD bullous pemphigoid (BP) antigen have been cloned.

Polymerase chain reaction (PCR) rapid amplification of cDNA ends (RACE) has been used to clone additional 5' cDNA sequence for BP antigen.

Sequence analysis indicates that BP antigen is similar in structure, charge periodicity, and amino acid sequence to desmoplakin I, a desmosomal plaque 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.

The diagnosis of both sporadic and drug-induced PF and PV can be made by immunoprecipitation of their respective antigens.

Paraneoplastic pemphigus is a new disease with distinctive clinical, histologic, and immunofluorescence findings. The antigens defined by autoantibodies in this disease are distinct from PV and PF antigens.

We have shown at a molecular level that PF and BP can occur in the same patient.

Publications:

Stanley JR. Is pemphigus an anti-adhering junction autoimmune disease?, J Dermatol Sci 1990;1:237-244.

Robledo MA, Kim S-C, Korman NJ, Stanley JR, Labib RS, Futamura S, Anhalt GJ. Studies of the relationship of the 230-kD and 180-kD bullous pemphigoid antigens, J Invest Dermatol 1990;94:793-797.

Stanley JR. Pemphigus: skin failure mediated by autoantibodies, JAMA 1990;264:1714-1717.

Rico MJ, Korman NJ, Stanley JR, Tanaka T, Hall RP. IgG antibodies from patients with bullous pemphigoid bind to localized epitopes on synthetic peptides encoded by bullous pemphigoid antigen cDNA, J Immunol 1990;145:3728-3733.

Anhalt GJ, Kim SC, Stanley JR, Korman NJ, Jabs DA, Kory M, Izumi H, Ratrie H, Mutasim D, Ariss-Abdo L, Labib RS. Paraneoplastic pemphigus. An autoimmune mucocutaneous disease associated with neoplasia, *N Engl J Med* 1990;323:1729-1735.

Korman NJ, Stanley JR, Woodley DT. Coexistence of pemphigus foliaceus and bullous pemphigoid, *Arch Dermatol* 1991;127:387-390.

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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* (in press).

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* (in press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03669-02 D

PERIOD COVERED

October 1, 1990 to September 30, 1991

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, D, DCBDC, NCI
Aimin Tang, M.D., Visiting Fellow, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)

William T. Golde, Ph.D., University of Colorado Health Sciences Center,
Denver, CO.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.58

PROFESSIONAL:

2.58

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

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

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

The major focus of this laboratory has been to determine how ultraviolet radiation (UV) modulates the epidermal immune system. Low doses of UV-B administered to epidermal cells (EC) in vitro completely inhibited the ability of freshly-isolated Langerhans cells (fLC) to support the mitogenic response of T cells to anti-CD3 monoclonal antibodies (mAb). Exposure of fLC to low doses of UV-B in vitro prevented LC-T cell interactions and upregulation of ICAM-1 expression by fLC. Anti-ICAM-1 mAb inhibited the proliferative response of T cells to anti-CD3 mAb supported by fLC, confirming that the ICAM-1/LFA 1 interaction is critical in this system. LC that had been cultured for 24-72 h (cLC) were functionally resistant to UV-B and expressed increased levels of ICAM-1. Because cLC express several important adhesion molecules, the functional resistance of cLC to UV-B may not result from increased ICAM-1 expression alone. cLC were functionally resistant to UV-B even though UV-B was cytotoxic to cLC. This apparent paradox was resolved when it was discovered that anti-CD3 mAb-induced T cell activation became irreversible within several hours after culture initiation, before UV-B-induced cytotoxicity became manifest. fLC caused T cells to become activated at a substantially slower rate. The enhanced accessory cell activity of cLC relative to fLC may be attributable to the fact that cLC express significant levels of several important adhesion molecules. UV-C and psoralen + UV-A had similar inhibitory effects on fLC function and ICAM-1 expression but markedly decreased LC survival in vitro. Re-examination of the effects of UV-B on LC survival revealed that although similar numbers of LC were recovered from cultures of irradiated and unirradiated EC 24 h after culture initiation, progressively fewer LC were recovered from cultures of irradiated EC at 48 and 72 h. These results indicate that low dose UV-B is lethal to LC in vitro and presumably also in vivo.

Cultured LC (cLC) were found to be functionally resistant to levels of UV-B that completely inhibited FLC accessory cell activity. Although we initially thought that cLC were resistant to UV-B radiation because they expressed high levels of ICAM-1, this may be an oversimplification. Blocking studies performed with mAb directed against adhesion molecules indicated that concentrations of anti-LFA-1 and anti-ICAM-1 mAb that blocked the ability of FLC to support anti-CD3 mAb-induced T cell mitogenesis caused only partial inhibition of cLC accessory cell function. Concentrations of anti-CD2 mAb which inhibited the activity of FLC (presumably by preventing the interaction of CD2 on T cells with LFA-3 on FLC) had no effect on cLC accessory cell function alone, but like anti-Mac-1 mAb augmented the partial inhibition observed with anti-LFA-1 mAb. Anti-LFA-1, anti-CD2 and anti-Mac-1 mAb in combination inhibited the functional activity of cLC by >90%. These results suggest that cLC are potent accessory cells in part because they express several functionally important adhesion molecules, only one of which is ICAM-1. It is therefore not possible to directly relate the functional resistance of cLC to UV-B radiation to expression of ICAM-1.

Our analysis of the effects of UV-B radiation on the accessory cell function of cLC was complicated by the observation that UV-B radiation was cytotoxic for cLC. Approximately two-thirds of the cLC recovered from 24 h cultures seeded with UV-B irradiated cLC were permeable to propidium iodide, whereas virtually all the cLC recovered from control cultures were viable. Utilizing a combination of mAb directed against adhesion molecules that was capable of preventing and reversing LC-T cell interactions, we demonstrated that T cells became committed to proliferate within the first several hours of culture initiation when cLC are used as accessory cells, while FLC induced T cell activation at a considerably slower rate. cLC appear to be functionally resistant to UV-B radiation because they express sufficient levels of adhesion or co-stimulatory molecules enabling irreversible T cell activation to occur before cytotoxic effects of UV-B become evident. In contrast, FLC must apparently acquire adhesion molecules (including ICAM-1) in culture before they become functionally active.

To begin to define the photoreceptor(s) responsible for the inhibitory activities of UV-B radiation and elucidate the mechanism(s) by which these activities are expressed, we examined the effect of several other types of UV radiation on LC accessory cell function, ICAM-1 expression and LC survival in vitro. UV-C radiation inhibited ICAM-1 expression and LC accessory cell activity, albeit at lower doses than UV-B radiation (with essentially complete inhibition occurring at 20 J/m²). UV-A radiation (0.25 J/cm²) inhibited LC ICAM-1 expression and function only if cells had been pre-treated with 8-methoxypsoralen. Careful examination of the effects of UV-C and psoralen + UV-A (PUVA) radiation on the survival of FLC in vitro revealed that although similar numbers of LC were recovered from cultures seeded 24 h earlier with unirradiated LC or LC that had been exposed to UV-C or PUVA, recovery of irradiated LC at 48 and 72 h was only 10% of that from cultures of unirradiated EC. Careful examination of the effects of UV-B radiation on the survival of FLC in vitro indicated that no cytotoxicity was evident at 24 h. Recovery of UV-B-irradiated LC was 50% of control at 48 h and 10% of

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control at 72 h, however. Because nucleic acids are known to be important photoreceptors for UV-B, UV-C and psoralen + UV-A radiation, it seems likely that modulatory effects of UV radiation on ICAM-1 expression, LC accessory cell activity and LC survival are mediated through UV-induced covalent modifications of DNA which inhibit gene transcription. This conclusion is supported by the results of experiments which indicate that low doses of actinomycin D (0.25 $\mu\text{g}/\text{ml}$) and UV-B radiation have similar selective effects on ICAM-1 expression and LC survival in vitro. (Time devoted to AIDS is 10%).

Publications:

Tang A, Udey MC. Inhibition of epidermal Langerhans cell function by low dose radiation: Ultraviolet B radiation selectively modulates intercellular adhesion molecule-1 (CD54) expression by murine Langerhans cells, J Immunol 1991;146:3347-3355.

Udey MC, Peck RD, Pentland AP, Schreiner GF, Lefkowitz JB. Antigen presenting cells in essential fatty acid deficient murine epidermis: Keratinocytes bearing class II antigens may potentiate the accessory cell function of Langerhans cells, J Invest Dermatol (in press).

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

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 AIDS and malaria. 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) Studies of the arrangement of the immunoglobulin and T-cell receptor genes. One focus in this area is the study of novel transforming genes that translocate into the T-cell receptor locus in T-cell malignancies. 6) Analysis of action of immunoregulatory cells including helper T cells, suppressor T cells, and macrophages that regulate antibody responses, and on studies of disorders of immunoregulatory cell interactions and of leukemias of these immunoregulatory cells. 7) 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

The molecular cloning of the lymphoid-restricted transcription factor, Oct-2 by Dr. Louis M. Staudt helped to define the POU-domain transcription factor multigene family. POU-domain transcription factors regulate gene expression in a variety of cell lineages during development. Oct-2 is expressed in B lymphocytes

and is critical for the lymphoid-specific expression of immunoglobulin genes. Most non-B cells in adult bone marrow express very little Oct-2 but as lymphoid progenitor cells differentiate into pre-B cells, Oct-2 expression is up-regulated. A unique regulatory role for Oct-2 in the T cell lineage is suggested by its induction during T cell activation by cognate antigen.

Another POU-domain transcription factor has been cloned and studied by Dr. Staudt, Oct-3, functions at the earliest stages of mammalian development. Oct-3 binds to the same DNA motif as Oct-2 yet has a distinct pattern of expression. Oct-3 is expressed in the pluripotent stem cells of early mammalian embryos and is then down-modulated when these cells become committed to more differentiated lineages. Oct-3 expression is, however, maintained in the germ cell lineage. The expression pattern places Oct-3 in marked contrast with all previously cloned transcription factors which are only detectable after stem cells have differentiated along one of the somatic lineages.

Dr. Staudt showed that the Oct-3 mRNA present in oocytes is transferred to the one-cell embryo and is absolutely required for embryonic DNA synthesis and cell division. Oct-3 is thus the first described mammalian maternal effect gene. Interestingly, little if any transcription takes place in one-cell embryos, raising the possibility that Oct-3 directly regulates chromosomal DNA replication.

SOMATIC GENE THERAPY FOR HUMAN GENETIC DISEASE

Dr. Michael Blaese's laboratory has continued to focus on the development of gene therapy. On September 14, 1990, his group performed the first authorized use of gene transfer to treat human disease by infusing 10^9 autologous ADA gene-corrected T cells into a 4-year-old girl with ADA deficiency SCID. Retroviral-mediated gene transfer was used to insert a normal functional human ADA gene into this girl's polyclonal peripheral blood T cells which had been stimulated to proliferate in tissue culture with an anti-T-cell receptor monoclonal antibody and IL2. The gene-corrected T cells were expanded 100-1000 fold in culture and were then returned intravenously within 2 weeks to maintain a polyclonal repertoire. This girl and a second ADA deficiency patient have been treated every 5-7 weeks with such gene-corrected T cell infusions and are now showing signs of enhanced immunological reactivity. Each now has a normal peripheral blood T cell count and each has begun to produce normal amounts of isohemagglutinins in response to environmental antigen stimulation. The first child has been skin tested and manifested a positive delayed hypersensitivity response to tetanus toxoid for the first time in her life. Extensive data on the quality of immune system function and the duration of the gene transfer effect are continuing to be collected and analyzed on these patients. The positive early results are encouraging the expansion of the protocol to include additional patients. Similar cellular immunorestitution protocols are being developed to treat patients with AIDS in the coming year.

MECHANISMS OF ANTIGEN-PRESENTATION AND T-LYMPHOCYTE RECOGNITION: APPLICATION TO VACCINE DESIGN

Dr. Jay Berzofsky has defined mechanisms by which T cells recognize antigens presented on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and has applied these insights to the design of synthetic vaccines for AIDS, malaria, and cancer. Dr.

Berzofsky has been able to quantitate the requirements for class I MHC molecules and for antigenic peptide for stimulation of CD8⁺ cytotoxic T lymphocytes (CTL) using purified class I molecules bound to plastic microtiter wells. He found that the titration curves for peptide and for MHC were interdependent, and that no accessory molecules were necessary. He also found that excess $\beta 2$ microglobulin is necessary for peptide binding, and conversely, peptide stabilizes association of $\beta 2\mu$ with class I heavy chains. In studying epitope recognition by HIV-specific CTL, Dr. Berzofsky demonstrated that human CTL from HIV-infected individuals recognized four peptides from the envelope protein in association with HLA-A2. One of these was a dominant CTL epitope in the mouse, but three had been identified originally as helper epitopes. Conversely, he found that the CTL epitope in the mouse was also recognized by helper T cells with class II MHC molecules to helper T cells. The extent of this concordance between helper and CTL epitopes and the molecular mechanism behind it is being explored. These dual function epitopes recognized by both helper T cells and CTL may be useful components of synthetic vaccines for HIV. To overcome this problem of MHC polymorphism in the human population, Dr. Berzofsky made peptides spanning multideterminant regions of HIV gp160 and found that these were recognized by mice of multiple MHC types and by T cells from a large fraction of humans. These are being incorporated in experimental vaccines. He also found that IL-2 production in response to gp160 peptides may be an earlier diagnostic for infection or at least exposure to HIV than antibody responses and possibly even PCR of peripheral blood cells. Thus Dr. Berzofsky's analyzing of the mechanism by which T cells recognize antigens is providing the scientific basis for the development of a rational subunit vaccine for AIDS as well as new approaches to diagnosis of HIV infection.

THE MULTICHAIN IL-2 RECEPTOR: MOLECULAR CHARACTERIZATION AND USE AS A TARGET FOR IMMUNOTHERAPY

Effective therapy of cancer using unmodified monoclonal antibody mediated therapy has been elusive. A number of factors explain the low therapeutic efficacy observed. Unmodified monoclonal antibodies are immunogenic and elicit a human immune response to the murine antibody. Moreover, mouse monoclonal antibodies are not cytotoxic against neoplastic cells in humans and in most cases are not directed against a vital cell surface structure such as a receptor for a growth factor required for tumor cell proliferation. Dr. Tom Waldmann has addressed these issues by using the IL-2 receptor as a target for monoclonal antibody immunotherapy, by genetic engineering to create less immunogenic and more effective monoclonal antibodies, and by arming such antibodies with toxins or radionuclides to enhance their effector action. Dr. Waldmann previously identified two peptides that bind IL-2: the 55 kD protein IL2R α chain reactive with the anti-Tac monoclonal antibody, and the 70/75 kD IL-2R β protein reactive with a monoclonal antibody termed Mik β 1. He proposed a multichain model for the high affinity receptor in which both IL-2R α - and IL-2R β -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. 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 18 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. In parallel studies a "humanized" version of MikB1 that blocks binding to the IL-2R β component has been generated by combining the complementarity determining regions of MikB1 with human immunoglobulin framework and constant regions. As with anti-Tac "humanized" MikB1 manifests antibody-dependent cellular cytotoxicity. Furthermore, the addition of "humanized" MikB1 that blocks the interaction of IL-2 with the IL-2R β subunit complements the anti-IL-2R α 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 cytotoxic agents wherein α - and β -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 β -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). At the doses used (5 and 10 mCi per patient) no toxicity was observed in 5 of 6 patients studied. Five of the six patients underwent a sustained, partial or complete remission following yttrium-90 anti-Tac therapy. Thus it is hoped that yttrium-90 chelated to "humanized" MikB1 will prove to be effective, relatively nontoxic agents for the treatment of an array of human leukemias.

BIOLOGY OF THE IMMUNE RESPONSE

Dr. David Nelson identified a soluble form of the IL-2R α component (Tac protein) of the human IL-2 receptor in the culture supernatants of activated T cells, B cells, and monocytes in vitro and in the sera of normal individuals in vivo. Dr. Nelson, using an ELISA technique he developed, found elevated levels of soluble IL-2R α in the sera of patients with hairy cell leukemia (HCL), human retroviral diseases including adult T-cell leukemia (ATL) and the acquired immune deficiency syndrome (AIDS). In ATL and HCL patients the serum level IL-2R α was indicative of tumor burden and favorable responses to therapy were associated with reductions in the serum level of IL-2R α . Elevations of IL-2R α in the serum were also indicative of allograft rejection episodes in patients receiving liver and heart-lung allografts. Patients with autoimmune diseases also had elevated levels of IL-2R α in the serum and joint fluids. The measurement of soluble IL-2R α in various body fluids is thus useful in monitoring certain neoplastic and immune-mediated events in vivo.

Taken together, the insights concerning the IL-2/IL-2 receptor system provided by the studies of Drs. Waldmann and Nelson are providing a new perspective for the diagnosis, definition of prognosis, and for the treatment of certain neoplastic diseases and autoimmune disorders and for the prevention of allograft rejection.

ISOLATION AND CHARACTERIZATION OF BIOLOGICAL RESPONSE MODIFIERS AND OLIGOSACCHARIDES THAT REGULATE HUMAN IMMUNE RESPONSES

Dr. Andrew Muchmore continues to have a fundamental interest in the functional role of defined carbohydrate structures in directly regulating the human immune response. Based on his studies with uromodulin which he purified, characterized and ultimately molecularly cloned from human pregnancy urine, he has characterized an entirely new class of immunoregulatory substances based on carbohydrate structure. A series of high mannose structures exhibit a broad range of immunoregulatory activities. He has expanded his source of these carbohydrate moieties and is examining their function utilizing three different approaches. The first involves structural studies using a combination of ion exchange chromatography, coupled with HPLC techniques. Dr. Muchmore's aim is to characterize the minimal structure required for biologic activity. The second is to characterize the in vitro and in vivo biologic activity of these compounds in a number of different models. The third approach examines the mechanism of action of these compounds at a molecular level. These studies demonstrate that defined glycopeptides are able to regulate immune activity both in vitro and in vivo. Interestingly these pure carbohydrate structures are able to directly regulate gene transcription by modulating the affinity and activity of transcriptionally regulatory proteins including the oncogene products c-fos and c-jun. These studies point the way toward a more basic understanding of factors responsible for gene regulation and hold the prospect of characterizing an entirely new class of pharmaceutically active compounds based on a unique chemistry. Dr. Muchmore's laboratory has also undertaken two new projects which represent an extension of their previous studies on Uromodulin. First unpublished data from Dr. Vacquier's laboratory in San Diego demonstrate that sea urchin sperm exhibit a major protein with greater than 70% identity to Uromodulin. The protein retains such homology that Dr. Muchmore's antiserum against human uromodulin cross-react with the sea urchin protein. Using a human testicular cDNA library and a lambda gt11 expression system, he is in the process

of trying to isolate the human analogue.

INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS

IGF-II Receptor: In order to focus on tissues or developmental stages in which the insulin-like growth factor II/mannose 6-phosphate (IGF-II/Man6P) receptor may play an important role, Dr. Peter Nissley examined the developmental expression of the receptor in the rat. In his previous work, Dr. Nissley showed that the receptor protein exhibited very marked developmental regulation in most tissues, being high during embryonic and fetal stages and then declining postnatally. Dr. Nissley also obtained preliminary evidence that this regulation occurred primarily at the level of receptor mRNA expression suggesting transcriptional control. He performed additional RNA extractions from fetal and postnatal tissues in order to perform a statistical comparison of mRNA levels in fetal and postnatal tissues. A nuclease protection assay was used for these measurements. With the exception of lung, the postnatal decline in receptor mRNA was highly significant in all of the tissues examined. These additional experiments support the conclusion that the control of receptor expression is at the level of mRNA. However in some tissues, such as lung, translational control may be important.

IGF-I Receptor: There is substantial evidence that many of the growth effects of both IGF-I and IGF-II are mediated by the IGF-I receptor, a member of the growth factor receptor family with intrinsic tyrosine kinase activity. However compared to the knowledge of receptor coupling of some of the members of this family, such as the PDGF and EGF receptor, work on the IGF-I receptor has lagged. Dr. Nissley has employed a human osteosarcoma cell line (MG63) to study receptor signaling by the IGF-I receptor. The advantage of this cell line is that IGF-I alone causes full growth stimulation and the cells can easily be growth arrested. Work of others has shown that growth stimulation by IGF-I in this cell line is mediated by the IGF-I receptor.

It was reported that in Balb/c 3T3 cells pertussis toxin blocked IGF-I caused a dose-dependent stimulation of DNA synthesis with a peak response (up to 30-fold stimulation) at 10 ng/ml. However pertussis toxin failed to inhibit IGF-I induced DNA synthesis. Lack of G protein involvement in the IGF-I receptor signaling pathway was also supported by the inability of GTP S to inhibit the binding of ^{125}I -IGF-I to MG63 membrane preparations. Dr. Nissley concludes that a pertussis toxin-sensitive G protein does not mediate the IGF-I-induced mitogenic response in MG63 cells.

Dr. Nissley has begun to characterize the IGF-I dependent phosphorylation of the IGF-I in MG63 cells as a first step in investigating signaling by the IGF-I receptor in this cell line. After metabolic labeling with ortho- ^{32}P phosphate and addition of IGF-I, cell lysates were purified by affinity chromatography on antiphosphotyrosine affinity columns and/or immunoprecipitation by anti-IGF-I receptor monoclonal antibodies. Since phosphorylated species migrate similarly to the IGF-I receptor on SDS-PAGE, a combination of both antiphosphotyrosine affinity chromatography and immunoprecipitation was used to demonstrate IGF-I-dependent phosphorylation of the IGF-I receptor.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB-04002-22 MET

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (30 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
Richard P. Junghans, Ph.D., M.D.	Biotechnology Fellow	MET, NCI
Jack Burton, M.D.	Biotechnology Fellow	MET, NCI
Angus Grant, Ph.D.	Staff Fellow	MET, NCI

(See next page)

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, NCI
Radiation Oncology Branch, NCI

Metabolism Branch

SECTION

DSDC, NCI, NCI, NCI, Bethesda, Maryland

TOTAL MAN-YEARS:

12

PROFESSIONAL:

10

OTHER:

2

CHECK APPROPRIATE BOX(ES)

X

 (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. Waldmann developed IL-2 receptor directed therapy for patients with leukemia. The scientific basis for this approach is provided by his observation that resting T cells do not express IL-2 receptors but receptors are expressed by the abnormal T cells of patients with lymphoma/leukemia, those with select forms of autoimmune disease, and individuals rejecting allografts. Dr. Waldmann proposed a multichain model for the high affinity IL-2 receptor involving two IL-2 binding proteins: a 55 kD (IL-2R α) protein and a 75 kD (IL-2R β) MikB1. 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 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 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. To enhance its effector function anti-Tac was armed with toxins and α - and β -emitting radionuclides. In a clinical trial of ^{90}Y anti-Tac in ATL patients, at the doses used (5 and 10 mCi ^{90}Y anti-Tac per patient) no toxicity was observed in 5 of the 6 patients with ATL studied. Five of the six patients with ATL underwent a sustained 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

Craig Tendler, M.D.	Biotechnology Fellow	MET, NCI
Verena B. Bier, M.D.	Fogarty Visiting Fellow	MET, NCI
Carolyn K. Goldman	Microbiologist	MET, NCI
Frank Hartmann, M.D.	Fogarty Visiting Associate	MET, NCI

Project DescriptionMajor Findings:

Effective therapy of cancer using unmodified monoclonal antibody mediated therapy has been elusive. A number of factors explain the low therapeutic efficacy observed. Unmodified monoclonal antibodies are immunogenic and elicit a human immune response to the murine antibody. Moreover, mouse monoclonal antibodies are not cytotoxic against neoplastic cells in humans and in most cases are not directed against a vital cell surface structure such as a receptor for a growth factor required for tumor cell proliferation. Dr. Waldmann has addressed these issues by using the IL-2 receptor as a target for monoclonal antibody immunotherapy, by genetic engineering to create less immunogenic and more effective monoclonal antibodies, and by arming such antibodies with toxins or radionuclides to enhance their effector action. Dr. Waldmann previously identified two peptides that bind IL-2: the 55 kD protein IL2R α reactive with the anti-Tac monoclonal antibody, and the 70/75 kD IL-2R β protein reactive with a monoclonal antibody termed Mik β 1. He proposed a multichain model for the high affinity receptor in which both IL-2R α - and IL-2R β -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. 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 18 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 for 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. In parallel studies a "humanized" version of MikB1 that blocks binding to the IL-2R β component has been generated by combining the complementarity determining regions of MikB1 with human immunoglobulin framework and constant regions. As with anti-Tac "humanized" MikB1 manifests antibody-dependent cellular cytotoxicity. Furthermore, the addition of "humanized" MikB1 that blocks the interaction of IL-2 with the IL-2R β subunit complements the anti-IL-2R α 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 β -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 β -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). At the doses used (5 and 10 mCi per patient) no toxicity was observed in 5 of 6 patients studied. Five of the six patients underwent a sustained, partial or complete remission following yttrium-90 anti-Tac therapy. It is hoped that yttrium-90 chelated to "humanized" anti-Tac 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 new perspective for the treatment of certain neoplastic diseases, autoimmune disorders and for the prevention of allograft rejection.

Honors and Awards:

- 1991 Artois-Baillet Latour Health Prize
- 1991 4th Annual Duke University Award for Excellence in Immunologic Research
- 1991 Landmarks in Allergy and Clinical Immunology Award in Clinical Immunology
- 1991 NIH Director's Award

Publications:

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Kozak RW, Lorberboum-Galski H, Jones L, Willingham MC, Malek T, FitzGerald DJP, Waldmann TA, Pastan I. IL-2-PE40 prevents the development of tumors in mice injected with IL-2 receptor expressing EL4 transfectant tumor cells. *J Immunol* 1990;145:2766-71.

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Wright JJ, Wagner DK, Blaese RM, Hagenruber C, Waldmann TA, Fleisher T. Characterization of common variable immunodeficiency: identification of a subset of patients with distinctive immunophenotypic and clinical features. *Blood* 1990;76:2046-61.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04015-2-MET

PERIOD COVERED

October 1, 1990 through September 30, 1991

TITLE OF PROJECT (20 characters or less. Title must fit on one line between the corners.)

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

COOPERATING INSTITUTION	W. French Anderson, M.D.	Chief	MHB, NHLBI
	Steven A. Rosenberg, M.D., Ph.D.	Chief	SB, NCI
	Gary Parenteau, M.D.	Medical Staff Fellow	CSB, NHLBI
	Gene Shearer, M.D.	Section Chief	EBB, NCI

LAB/BRANCH

Metabolism Branch

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

8

PROFESSIONAL:

4

OTHER:

4

CHECK APPROPRIATE BOXES)

<input checked="" type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	100%
<input checked="" type="checkbox"/> (a1) Minors			
<input type="checkbox"/> (a2) Interviews			

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

Michael Blaese's laboratory continues to focus on the development of gene therapy. On 9/14/90, his group 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-mediated gene transfer was 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 iv within 2 weeks to maintain a polyclonal repertoire. This girl and a second ADA deficiency patient have been treated every 5-7 weeks with such gene-corrected T cell infusions and are now each showing signs of reconstituted immune reactivity. Each child now has a normal peripheral blood T cell count and each has begun to produce normal amounts of isohemagglutinins in response to environmental antigenic stimulation. The first child has a positive, delayed hypersensitivity response to tetanus toxoid for the first time in her life. Extensive data on the quality of immune system function and the duration of the gene transfer effect are continuing to be collected and analyzed on these patients. Similar cellular immunoreconstitution protocols are being developed to treat patients AIDS in the coming year. 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, antibody production in these experimental animal models and has shown utility in treating several different autoimmune disorders including experimental autoimmune uveitis and adjuvant arthritis.

Project DescriptionMajor Findings:

A major effort of the Cellular Immunology Section for the past several years has been directed toward the development of techniques of gene transfer for application to clinical gene therapy. Much of the early work in this field has been directed toward the correction of genetic diseases containing defects in the cells originating in the lymphohematopoietic lineage. If the totipotent bone marrow stem cell could be corrected by gene transfer and then returned successfully to the patient, a continuous stream of "gene corrected" progeny cells should reach the periphery and cure the disease. In 1985 we showed that retrovirus-mediated ADA gene transfer in vitro would successfully correct the primary metabolic defect in T cell lines established from children with severe combined immunodeficiency (SCID) secondary to adenosine deaminase deficiency (ADA). We also demonstrated in irradiated monkeys that were reconstituted with autologous "gene-treated" bone marrow that primate bone marrow could be engineered to express foreign genes in vivo. In these experiments, monkey bone marrow was treated in vitro with a retrovirus vector capable of transferring the human ADA gene. The reconstituted monkeys did produce human ADA in their circulating blood cells, but the level of human enzyme produced was low and its persistence was relatively transient. Although these observations were the first demonstration of successful gene transfer into primates in vivo, they also clearly demonstrated that bone marrow gene therapy was not yet ready for clinical application. This problem remains to be solved and appears to be the result of a combination of factors complicated by incomplete knowledge of the biology of the totipotent bone marrow stem cell. The stem cell appears to reside primarily in a G₀ state and retroviral-vectors require that their target cell be actively synthesizing DNA for successful gene transfer and integration. Until these problems were solved, we elected to explore the use of alternative cells for 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 the T cell lines from patients with ADA deficiency could be cured by retrovirus-mediated gene transfer. T cells are readily available for 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 lymphnode 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. Safety studies in monkeys showed that wild type murine retroviruses were not acute pathogens in these animals and no untoward effects at all were observed in the recipients of retroviral vectors or cells modified by retroviral vector mediated gene transfer.

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. In this study we used retroviral-mediated transfer of the bacterial gene for neomycin resistance (neoR) to provide a unique DNA label for T cells that were being used in the immunotherapy of malignant melanoma. This approach 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. Treatment with tumor infiltrating lymphocytes (TIL) resulted in remission in 40% of patients who had failed previous standard therapy, but little was known about the characteristics of TIL which might correlate with a therapeutic response. Our study used the NeoR gene to label the cells 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 to 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, gene therapy. In our studies of children with ADA deficiency SCID, it was shown that unexpectedly we could grow 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. After a long regulatory review, we received final permission to treat 10 ADA(-)SCID patients with autologous T cells corrected by ADA gene transfer. 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 where they are expanded in number by 100-1000 fold while the ADA gene is inserted, and then reinfused intravenously. To date, the first patient has received 7 infusions and the second patient 4 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. The one child tested so far is also capable of expressing positive delayed hypersensitivity skin test reactivity for the first time in her life. 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 in number, and then reinfused into the patients. A series of different gene modifications are planned for these studies.

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. 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. 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 1 MHC determinants in a normal fashion which is related to instability of the cell surface complex consisting of antigen, MHC class 1, 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, skin, and tumor allograft rejection in rats. SA used as the sole immunosuppressive agent prolongs the survival of cardiac transplants in monkeys for days to weeks and in 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." Its mechanism of action is still unknown and has eluded serious attempts at its definition. Treatment with immunosuppressive doses of SA does not inhibit any as yet measured T cell activation antigen. 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:

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Kwan S-P, Lehner T, Hagemann T, Lu B, Blaese RM, Ochs H, Wedgwood R, Ott J, Craig IW, Rosen FS. Localization of the gene for the Wiskott-Aldrich syndrome between two flanking markers, TIMP and DXS255, on Xp11.22-xp11.3. Genomics 1991;10:29-33.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04016-18 MET

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Mechanism of Action of Insulin-like Growth Factors

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

PI: S. Peter Nissley	Senior Investigator	MET, NCI
Daisy De Leon	IRTA Fellow	MET, NCI
Wlodzimierz Lopaczynski	Fogarty Visiting Fellow	MET, NCI

COOPERATING UNITS (if any)

Diabetes Branch, NIDDK
Molecular, Cellular and Nutritional Endocrinology Branch, NIDDK

LAB/BRANCH

Metabolism Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

5.1

PROFESSIONAL:

3.0

OTHER:

2.1

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 performed additional RNA extractions from tissues in order to perform a statistical comparison of IGF-II/mannose 6-phosphate mRNA levels between fetal (20 day) and postnatal (20 day) tissues. mRNA levels were measured with a nuclease protection assay. With the exception of lung, the postnatal decline in receptor mRNA was statistically significant in all of the tissues examined. These additional experiments support our earlier conclusion that the control of expression of the IGF-II/M6P receptor is primarily at the level of mRNA in most tissues. In addition, by using a sense strand standard which we transcribed from a pGem 4 plasmid containing a 0.5 kbIGF-II/M6P receptor insert, we have measured the number of receptor mRNA copies in fetal heart, the tissue with the highest level of receptor (10^8 molecules/heart). We have examined the role of G proteins in signaling by the IGF-I receptor by examining the effect of pertussis toxin on stimulation of DNA synthesis by IGF-I in the human osteosarcoma cell line MG63. In six experiments, pertussis toxin (0.01 pg/ml to 1 ng/ml) failed to inhibit IGF-I induced DNA synthesis in MG63 cells. Lack of G protein involvement in the IGF-I receptor signaling pathway was also supported by the inability of GTP γ S to inhibit the binding of 125 I-IGF-I to MG63 membrane preparations. We have begun to characterize the IGF-I dependent phosphorylation of the IGF-I receptor in MG63 cells as a first step in investigating signaling by the IGF-I receptor in this cell line. After metabolic labeling with ortho[32 P]phosphate and addition of IGF-I, cell lysates were purified by affinity chromatography on anti-phosphotyrosine Agarose and/or immunoprecipitation by anti-IGF-I receptor monoclonal antibodies. Because of phosphorylated bands which migrated similarly to the IGF-I receptor on SDS-PAGE, a combination of both anti-phosphotyrosine affinity chromatography and immunoprecipitation was required in order to demonstrate IGF-I dependent phosphorylation of the IGF-I receptor.

Project Description

Major Findings:

Developmental expression of the rat insulin-like growth factor-II/mannose 6-phosphate receptor mRNA

In order to provide a statistical comparison between IGF-II/M6P receptor mRNA levels in fetal and postnatal tissues, additional RNA extractions were performed and mRNA levels measured by a nuclease protection assay. At least 3 separate RNA extractions were performed on 20 day fetal and 20 day postnatal tissues and in the case of lung and fetal limb/postnatal muscle, 5 and 8 RNA samples were obtained. Statistical comparison in an unpaired t-test showed the following p values: lung, 0.054; limb/muscle, 0.006; kidney, <0.001; intestine, 0.022; liver, 0.024; and brain, 0.002. Thus with the exception of lung, the comparisons between fetal and postnatal mRNA levels were significant. These developmental changes in receptor levels agree with the developmental changes in total tissue receptor protein levels that we determined earlier by quantitative Western blotting except that the changes in mRNA levels are not as dramatic as the change in receptor protein for some tissues such as lung. Our additional experiments support our preliminary conclusion that the control of expression of the IGF-II/M6P receptor is primarily at the level of mRNA for most tissues. It is of interest that the highest levels of receptor protein and mRNA are in fetal heart since in a recent report of absent IGF-II/M6P receptor in the mouse, fetal death occurred at day 15 and the fetuses exhibited generalized edema (heart failure?).

We transcribed a sense strand from the pGem 4 plasmid carrying a 0.5 kilobase fragment of the IGF-II receptor for use as a standard in the nuclease protection assay. The sense strand standard was quantitated by low level radiolabeling with ³²P-UTP, measuring the radioactivity in the transcribed sense strand, and calculating the amount of sense strand from the known U content. Using this standard we determined that there are 10⁸ receptor mRNA copies in a 20 day fetal heart.

The role of G proteins in insulin-like growth factor I stimulated DNA synthesis in MG63 human osteosarcoma cells

The human osteosarcoma cell line, MG63, has characteristics which make it particularly attractive for the study of the growth promoting action of IGF-I. MG63 cells multiply in serum-free medium with IGF-I as the only growth factor, and growth in the absence of IGF-I is minimal. Growth stimulation by IGF-I is mediated by the IGF-I receptor since the monoclonal antibody α IR-3, which blocks binding of IGF-I to the IGF-I receptor also blocks IGF-I stimulated cell proliferation. It was reported that in Balb/c 3T3 cells pertussis toxin blocked IGF-I stimulated DNA synthesis suggesting G protein involvement in the IGF-I signaling pathway. We tested this hypothesis in the MG63 cells. IGF-I caused a dose-dependent stimulation of ³H-thymidine incorporation into DNA, with a peak response (up to 30-fold stimulation) at 10 ng/ml. In 6 experiments, pertussis toxin (0.01 pg/ml to 1 ng/ml) failed to inhibit IGF-1 induced DNA synthesis in the MG63 cells. Lack of G protein involvement in the IGF-I receptor signaling pathway was also supported by the inability of GTP γ S to inhibit the binding of ¹²⁵I-IGF-I to MG63 membrane preparations. We conclude that a pertussis toxin-

sensitive G protein does not mediate the IGF-I induced mitogenic response in MG63 cells.

Insulin-like growth factor-I dependent phosphorylation of the IGF-I receptor in MG-63 cells.

We have begun to characterize the IGF-I dependent phosphorylation of the IGF-I receptor in MG63 cells as a first step in investigating signaling by the IGF-I receptor in this cell line. Serum-starved MG63 cells were metabolically labeled with ortho-[³²P]phosphate and IGF-I (100 ng/ml) was added in serum-free medium. After various times, the cell monolayers were lysed with buffer containing 1% Triton X-100 and phosphatase inhibitors. The cell lysates were purified by either affinity chromatography on anti-phosphotyrosine-Agarose or by immunoprecipitation by anti-IGF-I receptor monoclonal antibodies. Alpha IR-3 was generously provided by Dr. Steve Jacobs and 18E9 was developed in our laboratory by fusing 653 plasmacytoma cells with spleen cells from a mouse that had been immunized with pure IGF-I receptor from human placenta. Following anti-phosphotyrosine affinity chromatography, analysis by SDS-PAGE (nonreducing conditions) and autoradiography, showed two closely migrating bands of approximately 300 kDa. The lower band was much more intense and did not show IGF-I dependence. After further immunoprecipitation by anti-receptor monoclonal antibodies, the lower band was no longer visualized and the upper band was clearly seen to be IGF-I dependent. Monoclonal antibody 18E9 which was much weaker than α IR-3 in an immunoprecipitation assay with ¹²⁵I-IGF-I labeled receptor, was also much less effective in immunoprecipitating the 300 kDa receptor band. Similarly, if the cell lysates were subjected to immunoprecipitation alone, the IGF-I receptor could not be resolved from more intensely labeled bands in the vicinity of the 300 kDa IGF-I receptor band. We conclude that IGF-I dependent phosphorylation of the IGF-I receptor can be demonstrated in MG63 cells after a combination of anti-phosphotyrosine affinity chromatography and immunoprecipitation with anti-receptor antibodies.

Publications:

Burguera B, Werner H, Sklar M, Shen-Orr Z, Stannard B, Roberts CT, Nissley SP, Vore SJ, Caro JF, LeRoith D. Liver regeneration is associated with increased expression of the insulin-like growth factor-II/mannose-6-phosphate receptor. *Mol Endocrinol* 1990;4:1539-45.

Kiess W, Greenstein IA, Lee L, Thomas C, Nissley SP. Biosynthesis of the insulin-like growth factor-II (IGF-II)/mannose 6-phosphate receptor in rat C6 glial cells. The role of N-linked glycosylation in binding of IGF-II to the receptor. *Mol Endocrinol* 1991;5:281-91.

Kiess W, Thomas CL, Sklar MM, Nissley SP. β -galactoside decreases the binding affinity of the insulin-like growth factor-II/mannose-6-phosphate receptor for insulin-like growth factor-II. *Eur J Biochem* 1990;190:71-7.

Nissley SP, Kiess W, Sklar MM. The insulin-like growth factor-II/mannose 6-phosphate receptor. In: LeRoith D, ed. *Insulin-like growth factors: molecular and cellular aspects*. CRC Press 1991;111-51.

Nissley P, Lee L, Kiess W. Evidence against a role for insulin-like growth factor-II in the autonomous growth of rat 18,54-SF cells. *Mol Cell Endocrinol* 1991;75:213-19.

Nissley SP, Lopaczynski W. Insulin-like growth factor receptors. *Growth Factors* 1991; in press.

Yang YW-H, Robbins AR, Nissley SP, Rechler MM. The chick embryo fibroblast cation-independent mannose 6-phosphate receptor is functional and immunologically related to the mammalian insulin-like growth factor-II (IGF-II)/man 6-P receptor but does not bind IGF-II. *Endocrinology* 1991;126:1177-89.

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

PROJECT NUMBER

Z01 CB 04017-13 MET

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Biology of the Immune Response

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

PI:	David L. Nelson, 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

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch

SECTION

Immunophysiology

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

5.8

PROFESSIONAL:

4

OTHER:

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 and hairy cell leukemia, reductions in serum levels of sIL-2R correlated with responses to therapy. 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 (HIV-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 DescriptionMajor Findings:

The cell membrane receptor for the T-cell derived lymphokine, interleukin-2 (IL-2) is a multichain structure consisting of at least two 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-2R α , we have identified a soluble form of this molecule which is 10 kDa smaller than the cell surface form of IL-2R α and established an enzyme-linked immunoassay (ELISA) for the measurement of this molecule in serum.

Elevated levels of soluble IL-2R α 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. Reductions in sIL-2R α correlated with responses to therapy in patients with ATL and HCL. The measurement of sIL-2R α 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 responsive human tumor cell line has been derived from a patient with intestinal lymphangiectasia and a secondary immunodeficiency disease. 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:

- 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.
- Kozak RW, FitzGerald DJP, Atcher RW, Goldman CK, Nelson DL, Gansow OA, Pastan I, Waldmann TA. Selective elimination in vitro of alloresponsive T cells to human transplantation antigens by toxin or radionuclide conjugated anti-IL-2 receptor (Tac) monoclonal antibody immunotherapy. *J Immunol* 1990;144:3417-23.
- Kurman CC, Rubin IA, Nelson DL. Soluble interleukin-2 receptor (sIL-2R, Tac protein). In: Rose NR, ed. *Manual of clinical laboratory immunology*, in press.
- Nelson DL, Kurman CC, Notarangelo LD. The cells and molecules of the immune system. *Pers Pediatr*, in press.
- Nelson DL, Kurman CC. The human immune response: molecular recognition and immunoregulation. In: Burbaker DB, Kasprisin D, eds. *Apheresis in cellular immunotherapy and stem cell collection*. Arlington, VA: American Assoc of Blood Banks, in press.

- Rubin IA, Snow KM, Kurman CC, Nelson DL, Keystone EC. Serial levels of soluble interleukin-2 receptor in the peripheral blood of patients with rheumatoid arthritis: correlations with disease activity. *J Rheumatol* 1990;17:597-602.
- Rubin IA, Nelson DL. The soluble interleukin-2 receptor: biology, function and clinical application. *Ann Intern Med* 1990;113:619-27.
- Rubin IA, Galli F, Greene WC, Nelson DL, Jay G. The molecular basis for the generation of the human soluble interleukin-2 receptor. *Cytokine* 1990;2:330-36.
- Plebani A, Notarangelo ID, 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 ADB, eds. *Progress in immune deficiency III*. London:Royal Society of Medicine Services Limited, 1991;41-42.
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- Wagner DK, Wong HL, Gately MK, Nelson DL. Cellular source of soluble interleukin 2 receptors in serum of mice after recombinant interleukin 2 administration. *Cytokine* 1990;2:337-43.
- Young JB, Lloyd KS, Windsor NT, Cocanouer 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 Transpl*, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 04018-15 MET
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PERIOD COVERED
October 1, 1990 through September 30, 1991

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:	4 $\frac{1}{2}$	PROFESSIONAL:	4
		OTHER:	$\frac{1}{2}$

CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	"B" 100%
<input type="checkbox"/> (a1) Minors			
<input type="checkbox"/> (a2) Interviews			

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

Our laboratory continues to have a fundamental interest in the functional role of defined carbohydrate structures in directly regulating the human immune response. Based on our studies with Uromodulin which we purified, characterized and ultimately molecularly cloned from human pregnancy urine, we have characterized an entirely new class of immunoregulatory substances based on carbohydrate structure. A series of high mannose structures exhibit a broad range of immunoregulatory activity. We have expanded our source of these carbohydrate moieties and are examining their function utilizing three different approaches. The first involves structural studies using a combination of ion exchange chromatography, coupled with HPLC techniques. Our aim is to characterize the minimal structure required for biologic activity. The second is to characterize the in vitro and in vivo biologic activity of these compounds in a number of different models. The third approach examines the mechanism of action of these compounds at a molecular level. These studies demonstrate that defined glycopeptides are able to regulate immune activity both in vitro and in vivo. Interestingly these pure carbohydrate structures are able to directly regulate gene transcription by modulating the affinity and activity of transcriptionally regulatory proteins including the oncogene products c-fos and c-jun. These studies point the way toward a more basic understanding of factors responsible for gene regulation and hold the prospect of characterizing an entirely new class of pharmaceutically active compounds based on a unique chemistry. The laboratory has also undertaken two new projects which represent an extension of our previous studies on Uromodulin. First unpublished data from Dr. Vacquier's laboratory in San Diego demonstrate that sea urchin sperm exhibit a major protein with greater than 70% identity to Uromodulin. The protein retains such homology that our antisera against human uromodulin cross-react with the sea urchin protein. Using a human testicular cDNA library and a lambda gt11 expression system, we are in the process of trying to isolate the human analogue.

Project DescriptionMajor Findings:

- 1) The immunosuppressive oligosaccharides of uromodulin have been purified and structurally characterized.
- 2) A panel of related oligosaccharides have also been purified and structurally characterized.
- 3) These compounds have been shown to
 - a. block T cell responsiveness in vitro.
 - b. induce a delayed inflammatory response in vitro.
 - c. induce PGE² synthesis in vivo.
 - d. compete with binding to IL-1, IL-2 and TNF.
- 4) In vivo studies have shown species restricted activity in mice, guinea pigs and miniature swine.
- 5) In vitro studies now demonstrate that these mannose oligosaccharides interact with the transcriptional activator factors

Publications:

Muchmore AV, Decker JM, Shaw A, Wingfield PW. Evidence that high manose glycopeptides are able to functionally interact with recombinant tumor necrosis factor and recombinant interleukin-1. *Cancer Res* 1990;50:6285-90.

Muchmore AV, Sathyamoorthy N, Decker JM, Sherblom AP. Evidence for direct biologic activity of high mannose glycopeptides. *J Leukocyte Biol* 1990;48:457-64.

Sathyamoorthy N, Decker JM, Sherblom AP, Muchmore AV. Evidence that specific high mannose structures directly regulate multiple cellular structures. *Mol Cell Biochem* 1991;102:139-48.

Sherblom AP, van Halbeck H, Ballou CE, Decker JM, Sathyamoorthy N, Muchmore AV. Immunoregulatory activity of oligomannans and high mannose N-type glycopeptides. *Carbohydrate Research* 1991, in press.

Smagula RM, van Halbeek H, Decker JM, Muchmore AV, Moody CE, Sherblom AP. Pregnancy-associated changes in oligomannose oligosaccharides of human and bovine uromodulin (Tamm-Horsfall glycoprotein). *Glycoconjugate J* 1990;7:609-24.

Winkelstein A, Muchmore AV, Decker JM, Blaese RM. Uromodulin: a specific inhibitor of IL-1 in treated human T cell colony forests. *Immunopharmacology* 1990 20:201-5.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04020-14 MET

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Antigen-specific T-cell activation, application to vaccines for malaria and AIDS

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

PI: Jay A. Berzofsky, M.D., Ph. D.	Section Chief	Met, NCI
Toshiyuki Takeshita, M.D., Ph.D.	Visiting Fellow	Met, NCI
Richard England, M.D., Ph.D.	Medical Staff Fellow	Met, NCI
Donna Barnd, Ph.D.	IRTA Fellow	Met, NCI
Mutsunori Shirai, M.D., Ph.D.	Visiting Associate	Met, NCI
Marika Kullberg	Special Volunteer	Met, NCI

COOPERATING UNITS (if any)

Gene M. Shearer, Ph.D.	Section Chief	ETB, NCI
John D. Minna, M.D.	Branch Chief	NMOB, NCI
David Carbone, M.D.	Staff Fellow	NMOB, 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:

10.5

PROFESSIONAL:

8.5

OTHER:

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.)

We studied the mechanisms by which T cells recognize antigens presented on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and the application of these principles to the design of synthetic vaccines for AIDS, malaria, and cancer. We have been able to quantitate the requirements for class I MHC molecules and for antigenic peptide for stimulation of CD8⁺ cytotoxic T lymphocytes (CTL) using purified class I molecules on plastic. The titration curves for peptide and for MHC were interdependent; no accessory molecules were necessary; excess β -2 microglobulin is necessary for peptide binding; conversely, peptide stabilizes association of β -2 with MHC heavy chains. In studying HIV-specific CTL epitopes, we found that human CTL from HIV infected individuals recognized four peptides from the envelope in association with HLA-A2. One of these was a dominant CTL epitope in the mouse, but 3 had been identified as helper epitopes. Conversely, we found that the CTL epitope in the mouse also stimulated CD4⁺ helper T cells. To test the extent of this promiscuity of peptides for multiple MHC molecules, we studied three peptides in mice of 10 MHC types, and found that each was presented with several class I MHC molecules. Also class I-MHC presented CTL epitopes from HIV reverse transcriptase and the malaria circumsporozoite protein were also presented by class II MHC molecules to helper T cells. Use of this concordance between helper and CTL epitopes in vaccine design is being explored. To overcome the problem of MHC polymorphism in the human population, we showed that peptides spanning multideterminant regions of HIV gp160 were recognized by mice of multiple MHC types and by T cells from a large fraction of humans. We also found that IL-2 production in response to gp160 peptides may be an earlier diagnostic for infection or at least exposure to HIV than antibody responses. We have also begun to search for tumor infiltrating CTL specific for mutant oncogene products in human cancers and animal models, with the aim of developing peptide immunotherapy to induce such CTL.

Continuation Sheet for PHS 6040

Other Cooperating Units:

Louis H. Miller, M.D.	Section Chief	LPD, 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
Bernard Moss, M.D., Ph.D.	Lab Chief	LVD, NIAID
Mario Clerici, M.D.	Visiting Fellow	ETB, NCI
Sanjai Kumar, Ph.D.	Visiting Fellow	LPD, NIAID
Walter Weiss, M.D.	Lt. Cdr.	ID, NMRI
Stephen Hoffman, M.D.	Cdr.	ID, NMRI

Project DescriptionMajor Findings:

We have been studying the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, the factors that determine which antigenic structures are more likely to be recognized, and the application of these principles to the design of synthetic vaccines for AIDS, malaria and cancer. T cells recognize antigen after it has been proteolytically processed into fragments or unfolded forms which then associate with MHC molecules on another cell, called an antigen-presenting cell by virtue of this function. Almost any cell can present endogenously synthesized antigen with class I MHC molecules, but dendritic cells, macrophages, and B cells specialize in presenting exogenous antigen with class II MHC molecules. Each of these steps can influence which antigenic determinants are seen by T cells.

In the area of antigen presentation, we have been studying the quantitative requirements for presentation of peptide antigens to CD8⁺ cytotoxic T lymphocytes (CTL) by purified class I MHC molecules in the absence of live presenting cells. We have taken advantage of the fact that the immunodominant CTL epitope of the HIV envelope that we identified, called P18, is presented by the class I molecule D^d, which has been prepared in soluble form by recombinant DNA methods by David Margulies (NIAID). In collaboration with his lab, we have discovered that the purified D^d molecules coated onto plastic microtiter wells can bind P18 and stimulate CD8⁺ CTL lines and clones, as well as hybridomas in vitro. Since the plastic wells cannot be killed, we use as a readout of activation interferon-gamma production in the case of CTL lines and clones, and growth inhibition in the case of CTL hybridomas. We have been able to titrate both the amount of D^d on the plastic, and the amount of peptide, and can show that the titration curves for each vary as a function of the other. In general, the dependency on the density of D^d is steeper than the dependency on peptide concentration. Further analysis on the quantitative requirements for triggering the T cell is in progress. The current results also show that CD8⁺ CTL can be activated with only solid-phase MHC-peptide complexes in the absence of other cells and without the need for other accessory molecules such as LFA-1 and ICAM-1. The role of CD8 molecules that can bind the class I molecule is being explored. In the course of these studies, we observed that peptide did not

appear to functionally bind to the purified D^d in the absence of serum, but the serum requirement could be replaced by purified human β -2 microglobulin (the non-polymorphic light chain of class I MHC molecules). Although the purified D^d had a stoichiometric amount of mouse β -2 microglobulin already associated, as far as could be measured, the enhanced binding of peptide showed the same concentration dependence on the human β -2 microglobulin as did the exchange of human for mouse β -2 on the solid-phase D^d. Conversely, exchange of human for mouse β -2 microglobulin depended on the presence of peptide that could bind to the D^d. Therefore, the β -2 and the peptide each appeared to stabilize the binding of the other. The β -2 microglobulin was not merely creating empty class I molecules that could bind peptide by renaturing free class I heavy chains that had lost their associated β -2, because incubating with human β -2 microglobulin first, followed by a brief wash and then the addition of peptide did not facilitate the binding of peptide. Likewise, incubation with peptide first, followed by a wash and then β -2 also did not work. Both β -2 microglobulin and peptide had to be present simultaneously to obtain binding of either, at least at 37°C. We conclude that peptide and β -2 microglobulin stabilize each other's binding in a concerted fashion, and that there is no long-lived stable intermediate that can be detected at 37°C. These results are consistent with a requirement for dissociation of β -2 from heavy chain for peptide to bind, and then a requirement for β -2 to rebind to stabilize the peptide-MHC complex.

In the case of class II MHC molecules, we have synthesized a series of 42 variant peptides, each with a single amino acid substitution at one of 14 positions in a helper T cell epitope of myoglobin that is immunodominant in association with several class II molecules, with the goal of determining the molecular basis for the immunodominance. We have determined which residues affect peptide activity for stimulating several helper T-cell clones, and are in the process of defining which residues among these affect binding to the class II MHC molecules.

With regard to HIV-specific CTL, we have examined the response of human T cells from HIV-infected individuals, in collaboration with Gene Shearer's lab (NCI). We tested peripheral blood T cells for their ability to kill autologous Epstein-Barr Virus (EBV)-transformed B cells incubated with synthetic peptides corresponding to T-cell epitopes of the HIV-1 envelope that we had identified originally using murine T cells. We used both the immunodominant CTL epitope in mice, P18, and several peptides that had been identified as helper T-cell epitopes, T1, T2, and TH4.1. All four of these had been found to induce IL-2 production by T cells from infected humans as well. We found that all four of these peptides could serve as CTL epitopes, in contrast to a control peptide from the envelope protein, P23, and in contrast to a myoglobin peptide also used as a control. Eleven of 25 donors tested had CTL that killed autologous targets with P18, and but a comparable fraction were positive for the other peptides that had been identified as helper epitopes as well: 9/25 for T1, 12/25 for T2, and 7/25 for TH4.1. The killing was by CD3⁺ T cells, and required the presence of the appropriate class I MHC molecule, as shown in two ways. First, HLA-mismatched targets were found to be killed only if they shared certain class I HLA molecules, not class II molecules. In particular, for HLA-A2 positive individuals, killing occurred whenever the targets expressed HLA-A2, even if no other MHC molecule was shared. To confirm the restriction to HLA-A2, we used a target cell line HMy that was class I-MHC negative and a transfectant of this

line expressing only HLA-A2. The latter was killed by CTL from HLA-A2 HIV-infected individuals in the presence of each of the peptides, but the untransfected cells were not killed and neither was killed in the absence of peptide or by T-cells from HLA-A2 negative individuals, which could still kill autologous targets. These results indicate that all four peptides can be presented by HLA-A2. Since this human class I MHC molecule is highly prevalent in the human population, this finding may account for the widespread recognition of these peptides by outbred unrelated individuals, and may make these peptides more widely useful in a vaccine. Moreover, since an HLA-A2 negative infected individual had CTL that could kill targets with peptides if they shared HLA-A1 and B8, some of these peptides must also be presented by one of these other class I molecules.

Earlier data from our lab had indicated that the immunodominant CTL epitope of HIV-1 in mice, P18, could also be presented by the class II MHC molecule A^d to CD4⁺ helper T cells and would induce help for the induction of CD8⁺ CTL recognizing the same peptide with D^d. We had mapped residues in the core of the peptide as responsible for binding to the class I MHC molecule, and for interaction with the CTL receptor. It was therefore of interest to see if the same or different residues were responsible for presentation by the class II MHC molecule. Studies measuring IL-2 production by immune spleen cells or measuring proliferation by a CD4⁺ T-cell line specific for P18, and testing a series of substituted peptides, each with a single substitution at a different position in the sequence, indicated that the same core region was responsible for activation of CD4⁺ helper T cells as was required for recognition by CD8⁺ CTL. We are in the process of distinguishing between residues that interact with the MHC molecule and those that interact with the T-cell receptor, but progress has been slowed by the finding that P18 appears to bind only with low affinity to A^d, and so it has been difficult to detect competition by P18 analogues for binding of other peptides to A^d.

Because of these observations that an immunodominant CTL epitope of HIV-1 gp160, P18 (residues 315-329), was presented by class II MHC molecules to helper T cells as well as by class I MHC molecules to CTL, and that several peptides of gp160 identified originally as helper epitopes also sensitized targets for lysis by human CD8⁺ CTL, we asked in the mouse how broad a range of class I MHC molecules, if any, could present these peptides. Spleen cells from mice of 10 different MHC types immunized with recombinant vaccinia expressing gp160 of HIV-1 IIIIB were restimulated in culture with helper peptides T1 or HP53 (also known as TH4.1), or with P18, plus IL-2. Resulting effector cells were tested for lysis of autologous targets treated with the relevant peptide and transfectants or vaccinia-infected cells expressing the whole gp160 molecule. We found that P18 was presented by 5 different class I MHC molecules in the mouse. Helper peptide HP53 was also found to be presented by 4 different class I MHC molecules to CTL and T1 was presented by 2 class I MHC molecules to CTL. The CTL were CD8⁺ and CD4⁻, and killed targets expressing endogenous whole gp160 or pulsed with peptide. Overlapping and mutant peptides showed remarkable similarity in the core of the peptide required for CTL recognition of the peptide presented by distinct class I molecules, indicating that there are not merely adjacent or overlapping epitopes contained in the same peptide. However, more subtle differences in fine specificity of different class I molecules were found. We conclude that the 2 helper epitopes tested were also recognized by CD8⁺ CTL with

class I MHC molecules. One of these and the CTL epitope P18 showed a striking degree of promiscuity in the range of class I molecules that could present them. These promiscuous epitopes should be useful in a vaccine to induce CTL immunity in an MHC diverse population. The ability to also elicit T help may give them a dual role in such a vaccine.

The concordance between helper and CTL epitopes was further noted in mapping helper T epitopes in the HIV-1 reverse transcriptase molecule. We examined 21 overlapping peptides from the conserved N-terminal portion of reverse transcriptase, residues 8-202, for their ability to stimulate T cells from mice of five different MHC types immunized with recombinant reverse transcriptase. We mapped three peptides that induced T-cell proliferation, and one of these was identical to the one that we had previously mapped as an epitope presented by class I MHC molecules to CD8⁺ CTL. This site may also be useful in vaccines to induce both helper T cells and CTL, and may be all the more so because it comes from a relatively conserved region of a conserved protein, in contrast to the envelope protein. In addition, we have also observed this concordance between peptides inducing help and those inducing CTL for two malaria proteins, the circumsporozoite proteins of the human malaria *P. falciparum* and the mouse malaria *P. yoelii*. In each case, the CTL epitopes were a subset of the epitopes inducing class II-MHC restricted helper or proliferating T cells. The molecular basis of this concordance is under investigation.

With regard to helper T cells, we attempted to overcome the problem that any given peptide would be presented by only a subset of class II MHC molecules, and so would not be effective in the whole outbred human population. Based on our earlier screening of overlapping peptides from the HIV envelope, we had identified six multideterminant regions of the envelope protein, each containing overlapping or adjacent epitopes presented by different class II MHC molecules. We have now tested the hypothesis that synthetic peptides encompassing such multideterminant regions will be recognized by T cells of multiple murine histocompatibility types as well as by human T cells representing multiple HLA types. Six such peptides of 20-33 residues in length were synthesized, and tested for their ability to stimulate T cells from mice of four distinct histocompatibility types immunized with recombinant envelope protein rgp160, as well as from 42 HIV-infected humans of different HLA types. Results identify several such peptides that are broadly recognized by mice of four histocompatibility types and by 52-73% of those infected humans who still retain IL-2 productive responses to control recall antigens such as influenza A virus or tetanus toxoid. 86% of such infected donors tested against at least three peptides respond to at least one of the six peptides. Moreover, immunization of mice with these peptides elicits T cells that respond to whole gp160. These peptides therefore may be useful for both vaccine development in the broad human population, and diagnostic or prognostic applications. The most effective of these are now being coupled to neutralizing antibody epitopes and CTL epitopes and their efficacy in inducing antibodies and CTL is being determined.

In collaboration with Gene Shearer's lab at NCI, Carol Tacket at University of Maryland Vaccine Center, and Janis Giorgi at UCLA, we have studied the production of IL-2 in response to helper epitope peptides by T cells from HIV-seronegative individuals at risk for HIV exposure. One individual at low risk was initially seronegative and did not respond to any of the four peptides T1,

T2, TH4.1, or P18, but 4 months later became positive to two of the peptides, and 12 months later became positive to all four peptides. He remained positive to the four peptides, but seronegative, at month 16, and did not become positive by antibodies or by PCR until month 19. Thus, T-cell responses to peptides from the HIV envelope were detectable more than a year before infection was detected by antibody serology or by PCR. Similarly, in five individuals at high risk who were seronegative, we repeatedly observed specific T cell responses to these four peptides but not to control peptides, one from the HIV envelope and one from myoglobin. Many (>100) seronegative controls not at risk for HIV exposure did not respond to the peptides. One of the five individuals seroconverted, but the other four so far remain negative by both antibodies and PCR. The fact that they respond to all four peptides in a specific fashion clearly indicates specific HIV exposure. Without a positive PCR test, it is not possible to prove conclusively that these individuals are infected. It is possible that the virus is sequestered in lymph nodes and other lymphoid organs and is so rare in the peripheral blood that the sampling for PCR was inadequate. We conclude that the T-cell IL-2 production response to these peptides is a more sensitive test for exposure to HIV than antibodies or even PCR, and may be an earlier diagnostic test for infection than these as well, if these people are indeed infected. If they are not infected, then this is a demonstration that individuals can be exposed to sufficient HIV to induce a specific CD4⁺ T-cell response without developing an established infection. Either way, these observations should be of great interest. We are now arranging to test larger numbers of seronegative individuals at risk, and to study longitudinal samples of blood cells frozen over time on individuals who eventually seroconverted. If we can consistently detect T cell responses before seroconversion, and if the number of false positives and false negatives is low, this may be a very useful test for diagnosis and prognosis.

Finally, we have undertaken a major new project to apply the same approach to determine the feasibility of developing of a novel peptide immunotherapy for cancer, in collaboration with Dr. John Minna and Dr. David Carbone, NCI. Mutant oncogene products are well-defined abnormal proteins in many tumors that may serve as tumor antigens for CTL, which can recognize processed internal proteins with class I MHC molecules even if the oncogene product is not expressed in intact form on the surface of the tumor cell. We are determining whether there are CTL among tumor infiltrating lymphocytes that are specific for mutant oncogene products, particularly p53 and ras, in human and animal tumors. If so, then expansion of these by peptide immunotherapy might facilitate rejection of the tumor. If not, then the negative results will be informative about the mechanisms of tumor escape from immune surveillance. So far, David Carbone has sequenced the mutant p53 or ras genes in a number of tumors, and we have synthesized the corresponding peptides and are beginning to try to grow out peptide-specific CTL from human tumor specimens and peripheral blood. We are carrying out parallel studies with mouse and rat tumors, as well as immunizing rats and mice to try to induce CTL specific for mutant oncogene products.

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Patent Application:

Berzofsky JA, Hosmalin A, Clerici M, Germain RN, Shearer GM, Moss B, Pendleton CD. Peptides stimulating cytotoxic T cells immune to HIV RT. Filed March 9, 1990. Application No. 07/489,825.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB-04024-4 MET

PERIOD COVERED

October 1, 1990 through September 30, 1991

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

Control of Gene Expression in Lymphoid Development

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

PI: Louis M. Staudt, M.D., Ph.D.	Senior Staff Fellow	MET, NCI
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LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBDC, NCI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

10

PROFESSIONAL:

8

OTHER:

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.)

The molecular cloning of the lymphoid-restricted transcription factor, Oct-2, helped to define the POU-domain transcription factor multigene family. POU-domain transcription factors regulate gene expression in a variety of cell lineages during development. Oct-2 is expressed in B lymphocytes and is critical for the lymphoid-specific expression of immunoglobulin genes. Most non-B cells in adult bone marrow express very little Oct-2 but as lymphoid progenitor cells differentiate into pre-B cells, Oct-2 expression is up-regulated. A unique regulatory role for Oct-2 in the T cell lineage is suggested by its induction during T cell activation by cognate antigen.

Another POU-domain transcription factor that has been cloned and studied in our laboratory, Oct-3, functions at the earliest stages of mammalian development. Oct-3 binds to the same DNA motif as Oct-2 yet has a distinct pattern of expression. Oct-3 is expressed in the pluripotent stem cells of early mammalian embryos and is then down-modulated when these cells become committed to more differentiated lineages. Oct-3 expression is, however, maintained in the germ cell lineage. The expression pattern places Oct-3 in marked contrast with all previously cloned transcription factors which are only detectable after stem cells have differentiated along one of the somatic lineages.

We have shown that the Oct-3 mRNA present in oocytes is transferred to the one cell embryo and is absolutely required for embryonic DNA synthesis and cell division. Oct-3 is thus the first described mammalian maternal effect gene. Interestingly, little if any transcription takes place in one-cell embryos, raising the possibility that Oct-3 directly regulates chromosomal DNA replication.

Project DescriptionMajor Findings:

Oct-2 is a transcription factor expressed in B lymphocytes that binds the octamer DNA motif in immunoglobulin (Ig) promoters. The expression pattern of Oct-2 supports a role for Oct-2 in B cell development. Using a sensitive assay for Oct-2 mRNA expression based on the polymerase chain reaction, it was possible to detect very low levels of Oct-2 mRNA in a population of cells sorted from mouse bone marrow that contained progenitors for the B cell and myeloid lineages. When this population of progenitor cells was placed in Witte-Whitlock cultures in which B cell development takes place, Oct-2 mRNA was dramatically increased. In contrast, no change in Oct-2 expression was seen when the same progenitor cells were placed in Dexter cultures which support myeloid differentiation. Furthermore, pre-B cells sorted from mouse bone marrow or fetal liver expressed distinctly higher levels of Oct-2 than found in the bone marrow progenitor population. Mature splenic B cells expressed the highest levels of Oct-2. These findings demonstrate that high level Oct-2 expression correlates with commitment to the B cell lineage.

In the T lymphoid lineage, Oct-2 is expressed at low levels in CD4+ and CD8+ T cells isolated from human peripheral blood or from mouse lymph nodes. Interestingly, the levels of Oct-2 can be increased dramatically in certain normal T cell lines by activation with cognate antigen and antigen presenting cells. In one such T cell clone, antigen stimulation induces Oct-2 protein expression to the level found in mature B cells. The stimulation of Oct-2 occurs over a long time course, first appearing at 3 hours and plateauing at 9 hours after stimulation. Cyclosporin A blocks the induction of Oct-2 as does treatment with protein synthesis inhibitors. Oct-2 is most likely transcriptionally activated by "immediate early" transcription factors that are induced within 30 minutes of T cell activation. These findings suggest that Oct-2 may play a role in the long term changes in gene expression that accompany T cell activation.

The molecular cloning of Oct-2 helped to identify a new multigene family of transcription factors, the POU-domain family, that is characterized by two shared structural domains, a POU-homeodomain and a POU-specific domain. We have molecularly cloned and characterized a new member of this POU-domain family, termed Oct-3. Oct-3 was first detected as a DNA binding protein specific for the octamer DNA motif that is preferentially expressed in undifferentiated embryonal carcinoma (EC) and embryonic stem (ES) cells. Since several members of the POU-domain family of transcription factors bind to the octamer DNA motif, it was suspected that Oct-3 would belong to this family. The Oct-3 gene was cloned by probing EC cell cDNA libraries with DNA fragments derived from the POU-domain of Oct-2. Oct-3 is a transcription factor that can transactivate octamer-dependent reporter constructs in transient transfection experiments.

A role for Oct-3 in early mammalian development was suggested by its expression in undifferentiated EC and ES cell lines. ES cell lines are derived directly from the inner cell mass (ICM) of the mouse blastocyst and are true totipotent stem cells: if they are re-introduced into a murine blastocyst, they can give rise to all somatic and germ line lineages. EC and ES cell lines can

be induced to differentiate in vitro with retinoic acid and during this process Oct-3 is down-regulated. In contrast, other homeobox genes are expressed at low or undetectable levels in undifferentiated EC and ES cells and at high levels following differentiation.

Studies of EC and ES cell lines suggested a relationship between Oct-3 expression and a pluripotent phenotype. This correlation was extended by analyzing Oct-3 mRNA expression during mouse development. From the one cell stage to the morula stage, the embryonic cells are believed to be totipotent and all appear to express Oct-3. At the early blastocyst stage, the pluripotent cells that give rise to the embryo proper are located in the ICM and these cells express high levels of Oct-3. The ICM subsequently differentiates into two cell types: primitive ectoderm, which retains pluripotency, and primitive endoderm, which is committed to differentiate into extraembryonic structures. Oct-3 is abundant in the primitive ectoderm but is down-modulated in the primitive endoderm. The trophoblast of the early blastocyst initially expresses low levels of Oct-3 but as these cells differentiate into extraembryonic tissues, Oct-3 becomes undetectable. Following implantation, during the process of gastrulation, mesoderm is formed by differentiation from the pluripotent primitive ectoderm. Oct-3 is expressed at high levels in the primitive ectoderm but is down-modulated in the mesoderm. In 8.5 day embryos, Oct-3 is at low levels in ectodermal cells but after this time, Oct-3 is undetectable in any somatic cells. The down-modulation of Oct-3 expression during differentiation and loss of pluripotency in the embryo suggests that Oct-3 may be required to maintain a highly undifferentiated state.

An important clue to the function of Oct-3 comes from its expression in the germ line lineage. Primordial germ cells express Oct-3 throughout their migration from the allantois to the genital ridges. In the adult, Oct-3 is found in both the ovary and the testis. Within the ovary, Oct-3 is confined to oocytes, with maturing oocytes expressing distinctly higher levels than resting oocytes. The cells expressing Oct-3 in the testis have not yet been defined, although neither Sertoli cells nor mature spermatozoa have detectable Oct-3.

The expression of Oct-3 in oocytes suggested that maternally-derived Oct-3 might regulate early zygotic development. In many organisms, mRNA present in the oocyte is used by the embryo prior to the onset of transcription from the embryonic genome. In *Drosophila*, maternal effect genes are critical for the establishment of the anterior/posterior and dorsal/ventral axis. In the mouse, maternally-derived mRNA is currently believed to be required for development to proceed to the two cell stage, at which time the zygotic genome becomes transcriptionally active.

The role of maternally-derived Oct-3 mRNA in mouse development was addressed by injecting antisense Oct-3 oligonucleotides into one cell embryos. The antisense Oct-3 oligonucleotides target Oct-3 mRNA for specific degradation, probably by an RNaseH-mediated mechanism. The loss of Oct-3 mRNA resulted in an arrest in development at the one cell stage. This developmental block could be reversed by coinjecting in vitro-synthesized Oct-3 mRNA. Oct-3 mRNA containing a frame shift mutation was unable to rescue the antisense Oct-3-induced block. Thus, Oct-3 protein is required to traverse the first embryonic cell cycle. Interestingly, injection of antisense Oct-3 oligonucleotide into one cell

embryos just prior to mitosis failed to block the first cell division but did arrest development at the two cell stage. This result demonstrates an additional requirement for Oct-3 during the second embryonic cell cycle.

The requirement for Oct-3 in one cell embryos is particularly surprising in light of previous studies suggesting that transcription of the mouse genome begins at the two cell stage. Little transcription of the embryonic genome can be detected in one cell embryos. Most importantly, treatment of one cell mouse embryos with high concentrations of alpha-amanitin, an inhibitor of RNA polymerases II and III, fails to block the first cell division but does arrest development at the two cell stage. Thus, if the critical role for Oct-3 in one cell embryos is as a transcription factor, then the prevailing dogma that embryonic transcription begins at the two cell stage would have to be revised. Clearly, any Oct-3 mediated transcription in one cell embryos would have to be at a very low level and would apparently be mediated by an RNA polymerase other than RNA polymerase II or III.

A provocative alternate hypothesis is that Oct-3 regulates cellular DNA replication in one cell embryos. This possibility was predicated on studies showing regulation of adenovirus DNA replication by an octamer motif. In support of this hypothesis, antisense Oct-3 oligonucleotide into one cell embryos led to an profound (87%) inhibition of DNA replication. The injection of DNA fragments containing the octamer motif into one cell embryos also blocked DNA replication suggesting that Oct-3 may control DNA replication by binding to octamer motifs in chromosomal DNA. In many cells, mitosis is tightly coupled to the completion of DNA replication. Therefore, one simple scenario is that loss of Oct-3 leads to a primary inhibition of DNA replication which causes a secondary block in cell division. An analysis of Oct-3 mutants and their ability to stimulate replication in one cell embryos may provide insight into the control of mammalian chromosomal DNA replication.

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SUMMARY REPORT
EXPERIMENTAL IMMUNOLOGY BRANCH
October 1990 - September 1991

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) transplantation biology; 7) tumor immunology; and 8) 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. 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 and assembled TCR complexes, and that intra-thymically generated CD4 signals regulate the TCR degradation rate in CD4⁺CD8⁺ thymocytes (9268).

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 identifiable 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 β 8 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 (9273).

Dr. Henkart's laboratory has studied the differentiation of resting CD8⁺CD4⁻ thymocytes to become cytotoxic in short term in vitro culture after stimulation with immobilized antibodies against the T cell receptor (9263). In the presence of IL4 and other lymphokines, strong proliferation and cytotoxicity was observed after 3 days. The expression of genes for granule components associated with cytotoxicity as well as other genes expressed by stimulated CD8⁺ cells have been analyzed. The lymphokine-mediated increase in cytotoxic activity is accompanied by an increase in expression of the granule proteins cytolysin, granzymes A and B, and the serglycin proteoglycan core protein. In contrast, these conditions cause equal or lesser amounts of message for the lymphokines γ -IFN, TNF, LT, and TY-5 compared to control cultures. If the activated CD8⁺ lymphocytes are re-exposed to surface-bound antibodies against the T cell receptor, they undergo a form of activation-induced cytotoxic response resulting in a metabolic decrease and death (9263). During these studies a RT-PCR system was established to analyze the expression of CD45 isoforms. Careful analysis of PCR products revealed significant amounts of a previously undescribed mRNA species in which exons 4,5,6, and 7 are all deleted. This message appears to be made to some degree by all lymphocytes analyzed and results in a very short region of extracellular ser/thr/pro-rich domain where most of the carbohydrate is attached (9263). Dr. Henkart's laboratory has collaborated with the Surgery Branch, NCI to study human tumor-infiltrating lymphocytes (TIL) for expression of granule protein mRNA using Northern blots. They found that most TIL express mRNA for cytolysin, granzyme A, granzyme B and serglycin and are cytotoxic when tested in "redirected" assays in which MAb against the T cell receptor triggers the cytotoxicity. Occasional TIL are not cytotoxic and show poor expression of the cytolysin while expressing at least some other granule genes (9263).

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) V β 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 V β expression was carried out by quantitating mRNA corresponding to the 19 V β gene families expressed in mice, as well as with monoclonal antibodies specific for 14 of the 22 individually expressed V β products, in a large panel of inbred strains. Strain-specific deletions in 12 of the 22 V β products were detected and were shown to be related to the expression of multiple MHC and non-MHC self determinants. These findings indicate that maintenance of tolerance to a variety of self determinants results in substantial deletions in the available TCR V β repertoire (9265). The self determinants that function as ligands for V β -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 V β deletion, such that any one of two or more unlinked genes was permissive for deletion. Ligands responsible

for deletion of V β 11- and V β 12-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.

The role of the thymus in TCR negative selection was analyzed in Dr. Hodes' laboratory by examining TCR V β expression in T cells which have matured in congenitally athymic nude mice. It was found that deletions of V β 3 and V β 11 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 (9265).

The role of endogenous lymphokines and the regulation of lymphokine gene expression during T cell activation have also been evaluated in Dr. Hodes' laboratory (9205). The activation of type 2 T helper clones either by anti-TCR (CD3) antibody or by interleukin 2 (IL2) resulted in proliferation. The proliferation induced by anti-CD3 was inhibited by antibody specific for the lymphokine interleukin 4 (IL4), which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, both anti-CD3 and IL2 induced the expression of a number of protooncogenes (e.g. c-myc, c-myb) and lymphokines (IL5). In contrast, anti-CD3 but not IL2 induced expression of IL4 and GM-CSF, indicating that regulation of IL4 expression is independent of that of IL5. The independent regulation of these lymphokines was further supported by the demonstration that cyclosporin A completely inhibited anti-CD3-induced expression of IL4 but not IL5, whereas inhibitors of protein synthesis completely inhibited expression of IL5 but not IL4.

The TCR expressed by clones specific for Staphylococcal nuclease (NASE) have been analyzed in Dr. Hodes' laboratory to determine the relationship between antigenic fine specificity and TCR expression (9258). Preferential expression of specific V α and V β products was observed, e.g. expression of V β 4 by six of seven independent clones specific for NASE peptide 91-110 in association with Aa^bA β ^b, and expression of V β 10 in five of nine independent clones specific for NASE 81-100 in association with Ea^kE β ^k. Conservation of receptor usage was also reflected in junctional sequence analysis by PCR amplification of α and β chain cDNA. TCR fine specificity for specific NASE determinants is therefore reflected in a restricted TCR repertoire in this response.

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, including analysis of β 1 integrins, CD31 and the ELAM-1 ligand. Detailed analysis of phenotypic heterogeneity among peripheral blood CD4⁺ T cells, and more recently CD8⁺ T cells, illustrates marked complexity of regulation of surface phenotype such as: 1) CD4 memory cells can be

subdivided into two subsets based on quantitative differences in expression of the CD45RB isoform; 2) memory cells seem to be much less abundant among CD8 cells than CD4 cells; and 3) VLA-4 stands out as an important parameter of differentiation both among CD4 cells and among CD8 cells. Analysis of the enormously complex phenotypes of peripheral T cells has prompted Dr. Shaw's laboratory to propose a model of peripheral T cell differentiation in which naive T cells are of relatively uniform phenotype but their activation in multiple different microenvironments gives rise to a wide variety of differentiated memory cell phenotypes.

Dr. Gene Shearer's laboratory has investigated a series of murine models of autoimmunity, including those with thyroiditis-like and with lupus-like characteristics (9282). These models are characterized by autoantibody production and activation of suppressor T cells that are selective in their effect on CD4⁺ T helper cells. Preliminary studies in humans with lupus indicate that most of these patients exhibit a selective defect in CD4⁺ T helper function. These studies may provide a better understanding of immune regulation and dysregulation in autoimmune disease.

Dr. Shearer's laboratory has also investigated the effects of different immunologic insults on the development of murine T lymphocytes from bone marrow stem cells (9264,9255). These studies indicate that either a GVH reaction or treatment of mice with cyclosporin A (CsA) results in thymic damage such that after irradiation and bone marrow grafting, both CD4⁺ and CD8⁺ cells develop, but there is a selective absence of CD4⁺ T helper function. If bone marrow transplantation precedes treatment with CsA, mice fail to develop single positive (CD4⁺CD8⁻ or CD4⁻CD8⁺) T cells. These results provide a model that should be useful for investigating: a) the role of the thymus in T cell development; b) the expression of T cell receptors; and c) the effects of GVH and CsA on immune reconstitution in patients exposed to these immunologic insults.

Studies of T lymphocyte responses in HIV⁺, asymptomatic individuals by Dr. Shearer's laboratory indicate that 75-80% of these patients exhibit selective CD4⁺ T helper cell (Th) functional defects without any AIDS symptoms and without loss of CD4⁺ cell numbers (9267). A defect of antigen-presenting cell (APC) function was not observed in any such asymptomatic individuals, although two distinct types of APC defects were detected in AIDS patients. The defect in CD4⁺ Th function was associated with suppressor T cells that produced a soluble factor that was also capable of selectively suppressing Th function. Dr. Shearer's laboratory has developed a very sensitive Th assay that permits the detection of HIV exposure and/or infection three-to-fourteen months before detection by the antibody or polymerase chain reaction assays. This sensitive assay has also been used to detect: a) subtle immunologic changes induced by antiviral drug therapy; and b) HIV-specific, Th immunization in volunteers immunized with a trial AIDS vaccine. The results of this study: a) permit the detection of multiple and sequential stages of T helper dysfunction not previously defined by AIDS diagnostic tests which are predictive for AIDS progression; b) implicate an APC defect in AIDS patients but not early in disease progression; c) provide an assay for diagnostic and immunotherapeutic evaluation in HIV⁺ patients; and d) suggest that an AIDS vaccine is possible.

Dr. Shearer's laboratory has also investigated cell-mediated immune function defects in patients with the Wiskott-Aldrich (W-A) primary immune deficiency, and has observed a series of T lymphocyte and antigen presenting cell defects similar to those found in HIV-infected individuals. In addition, B cell lines from the W-A patients were unable to serve as HLA class I self-restricted targets for cloned cytotoxic effectors, if they were incubated with peptide antigens at 37°C. However, the W-A patients' cells worked well as targets if they: a) were pulsed with peptide at 28°C instead of at 37°C; b) were infected with live virus at 37°C; or were incubated at 37°C with peptide that is recognized in association with HLA class II.

S. Sharrow's laboratory (9255) has utilized three-color immunofluorescence and cluster analysis techniques to identify subpopulations of murine thymocytes which are not easily distinguishable using conventional histogram analysis methodology. These cells express high levels of cell-surface T cell receptor and although positive for both CD4 and for CD8, express CD4/CD8 phenotypes distinct from that of most immature CD4⁺CD8⁺ thymocytes. These subsets of thymocytes thus share phenotypic characteristics of both immature and mature intra-thymic T cells and are thought to represent a transitional intermediate in T cell differentiation.

2. CELL BIOLOGY OF IMMUNE RESPONSES

The expression and function of cell adhesion molecules by B cells was analyzed by Dr. Hodes' laboratory (9266). The supernatants of type 2 T helper cells induced resting B cells to express altered cell surface phenotype. In particular, a subpopulation of B cells expressed increased levels of CD44/Pgp-1, a molecule associated with specific cellular adherence to extracellular matrix. When the effects of individual lymphokines were examined, it was demonstrated that recombinant IL5 induced a population of B cells expressing the unique phenotype CD44 bright, B220 (CD45) dull, class II (Ia) dull. Sorting experiments demonstrated that this population contained the bulk of proliferative and antibody secretory activity in the activated B cells. The subset of CD44 bright B cells bound specifically to hyaluronate, and this binding was specifically inhibited by anti-CD44 antibody. The CD44 molecules expressed on IL5-stimulated B cells migrate with a lower molecular weight than does CD44 expressed by control B cells, reflecting differential glycosylation. Thus, CD44 expressed on activated B cells may serve as an adhesion molecule playing a role in B cell trafficking in vivo, and its adhesive function may be regulated at the level of quantitative expression as well as qualitative differences in the expressed molecules.

A series of mAb was generated in Dr. Hodes' laboratory by immunizing rats with activated mouse B cells. One of these mAb (GL7) reacts by flow cytometry with a subpopulation of B cells activated with stimuli including LPS or anti-Ig, but does not react with resting B cells or with other lymphoid cells (9266). This mAb precipitates a 29-31 KDa molecule from activated B cells which appears to represent a previously undescribed activation-specific molecule.

Dr. Shaw's laboratory has been identifying and characterizing the functions

of cell surface molecules which facilitate T cell recognition. Particular progress has been made in understanding the molecular basis of T cell interactions with endothelium, which is critical to T cell recirculation and migration. Two new molecular pathways are being elucidated. A subset of memory T cells bind to the inducible ligand ELAM-1 on endothelial cells; since this pathway does not require prior T cell activation, it may be of primary importance in the initial attachment of memory T cells to inflamed endothelium *in vivo*. Dr. Shaw's laboratory has also defined many characteristics of the molecule CD31 on a subset of T cells which make it a very attractive candidate for regulating T cell adhesion to endothelium. Not only does CD31 mediate adhesion, but it also powerfully induces adhesion by the multiple integrins which are present but relatively nonadhesive on resting T cells. As such, it appears to be a critical element in a T cell adhesion cascade. T cell interaction with endothelium via three other molecular pathways: VLA-4/VCAM-1, LFA-1/ICAM-1 and LFA-1/ICAM-2 has also been systematically analyzed. The relative importance of these multiple pathways depends critically on the state of activation of the T cell and of the endothelium as well as on the subset of T cells.

Studies from Dr. Shaw's laboratory have highlighted the importance of regulation of adhesion to understanding T cell function. Not only CD3 and CD31, but also the T cell surface molecules CD7 and CD28 can augment the adhesive function of multiple integrins expressed by T cells. The adhesive capacity of T cells changes with differentiation not only as a result of changes in the level of expression of adhesion receptors but also by changes in the expression of these molecules which regulate function of the adhesion receptors. It is noteworthy that CD31 and CD7 are preferentially expressed on naive cells and facilitate naive cell adhesion, in contrast to previous molecular pathways which have favored memory cell adhesion.

Cell-mediated cytotoxicity against tumor cells generally requires not only receptors on the effector cells that recognize tumor cells and trigger the release of cytotoxic effector molecules, but also effector cell activation that provides a cytotoxic mechanism accessible to a triggered response. Studies from Dr. Wunderlich's laboratory have identified and analyzed a new way for activating both MHC-independent cell-mediated cytotoxicity and targetable T-cell cytotoxicity against tumor cells (9250). They found that a 5-day exposure of mouse splenocytes to nanogram levels of native or attenuated bacterial lipopolysaccharide (LPS), stimulates antitumor activity of T cells targeted with bispecific antibodies. Freshly explanted splenocytes from normal donors lack this activity. With the addition of selected polyanions, MHC-nonrestricted antitumor cytotoxic activity also increases. The cellular basis for the LPS-induced response involves three types of cells: precursors to the cytotoxic cells, helper T cells, and accessory cells that are primarily radiation-sensitive B cells. LPS acts largely on the B cells. The helper cells are primarily CD4⁺ T cells. CD8⁺ T cells, however, can also serve this role, which may be important in situations where CD4⁺ cells have been incapacitated by infections such as HIV. Under normal circumstances, this mechanism may be initiated *in vivo* by LPS-activation of B cells in the intestinal wall.

3. SIGNAL TRANSDUCTION

The role of "second messengers" mediating activation of T cells through the TCR/CD3 complex was analyzed in cloned T cell populations by Dr. Hodes (9281). A cloned T cell population that was maintained by repeated stimulation in vitro with IL2 alone was capable of responding to subsequent stimulation with anti-CD3 antibody by proliferating and by strong phosphatidyl inositol (PI) hydrolysis and increased intracellular calcium concentration. In contrast, the same cloned line maintained by stimulation with specific antigen and antigen-presenting cells responded to anti-CD3 stimulation by proliferating, but without measurable PI hydrolysis or calcium response. The ability to transduce a TCR-mediated signal through the PLC pathway in cloned T cells is therefore influenced by prior stimulation through the TCR.

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.

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. Of particular importance, they have demonstrated that interaction of T cell VLA-4 with its ligand VCAM-1 provides a powerful costimulus to T cell activation, which complements others' observations that VCAM-1 is present in vivo on certain critical antigen presenting cells, such as tissue macrophages.

Dr. Weissman's laboratory has been interested in the T cell antigen receptor ζ (zeta) subunit, which is a key structure in receptor mediated signal transduction (9292). The ζ subunit is a substrate for a receptor activated protein tyrosine kinase and undergoes multiple, apparently cooperative, phosphorylations in response to receptor activation. As a consequence of this the electrophoretic mobility of ζ is altered from 16 to 21kD on reducing SDS/PAGE electrophoresis. In order to dissect out which tyrosines of ζ are phosphorylated and to understand the functional consequences of these modifications, a systematic mutational analysis of the ζ subunit was undertaken. In this study each of the 6 intracellular

tyrosines was mutated to phenylalanine. While mutation of either tyrosine 72 or 83 had little effect, mutation of tyrosines 123, 142 and 153 results in marked abnormalities in the migration of phosphorylated ζ suggesting that these tyrosines are involved in a co-operative process. Mutation of tyrosine 111 results in loss of all detectable phospho- ζ suggesting that this residue is particularly crucial in the generation and or maintenance of ζ subunits with multiple phosphorylation states. The results of these studies underscore the co-operative nature of tyrosine phosphorylation of this substrate. The functional consequences of these mutations was assessed by analysis of IL-2 production and phosphoinositol production. In 2B4 hybridoma cells there is no evidence of qualitative abnormalities in IL-2 production that can be correlated with the loss of ζ phosphorylation. Further studies are currently being planned to assess the role of phospho- ζ by the generation of transgenic animals containing the mutation at residue 111.

In order to dissect the molecular mechanisms responsible for coupling receptor occupancy to signalling pathways Dr. Weissman's laboratory has developed a permeabilized cell system for the study of T cell hybridomas (9292). In this system hybridoma cells can be permeabilized and still maintain the capacity to carry out such functions as tyrosine phosphorylation and phosphoinositide hydrolysis. Treatment of permeabilized cells with the tyrosine phosphatase inhibitor sodium orthovanadate resulted in a substantial enhancement of the amount of ζ that is tyrosine phosphorylated in response to receptor stimulation. In the absence of external stimulation there was no evidence for phosphorylation even in the presence of vanadate, suggesting that in the basal unstimulated state there is little turnover of phosphates on this subunit. Treatment of cells with the non-hydrolyzable GTP analogue GTP γ S results in an enhancement of receptor stimulated phosphorylation. This suggests that GTP binding proteins may be involved in the modulation of tyrosine kinase and or tyrosine phosphatase activity. Further studies are underway to expand these observations to other cells, to characterize GTP binding proteins that may play a role in this process and to assess the possibility that ζ itself may be a GTP binding protein.

Dr. Weissman's laboratory has also examined the signalling role of the ζ chain in natural killer cells, which are CD3 negative (9292). His laboratory has been able to demonstrate that the ζ subunit is physically associated with the Fc receptor in these cells. Stimulation of purified natural killer cells via the Fc receptor resulted in the tyrosine phosphorylation of ζ . Analogous to T lymphocytes, this phosphorylation resulted in a change in migration of this subunit such that it migrated at 21kd in reducing SDS/PAGE gels. This tyrosine phosphorylation is stimulation specific in that it did not occur in response to interleukin-2 stimulation or to treatment of cells with phorbol esters and ionomycin. These results demonstrate that ζ is physically associated with the Fc receptor on natural killer cells and that, as in the case of the T cell receptor, stimulation via the Fc receptor has as a readout the tyrosine phosphorylation of ζ .

4. STRUCTURE, REGULATION AND FUNCTION OF GENES INVOLVED IN IMMUNE RESPONSES

The MHC class I molecules, which serve as the targets of cellular immune responses and allograft rejection, are expressed on nearly all somatic tissues. However, their level of expression varies markedly among the tissues. In order to investigate the molecular basis for the differential patterns of expression, the laboratory of Dr. Dinah Singer has begun to identify and analyze regulatory DNA sequence elements and to generate a series of transgenic mice containing variants of the class I regulatory domain (9270). A series of negative and positive regulatory DNA sequence elements within the 5' flanking region of a gene, PDI, encoding a classical transplantation antigen have been identified. One regulatory complex has been identified which consists of overlapping negative and positive elements. Each element is associated with distinct cognate trans acting factors. Dr. Singer's laboratory has further observed that the level of factors binding to the silencer element are inversely proportional to the level of class I gene expression; enhancer binding factor is observed in all cells. These findings have led to the proposal that class I genes are negatively regulated and that tissue-specific levels of gene expression result from an equilibrium between the activities of the negative and positive elements associated with the complex. Introduction into transgenic mice of a series of nested deletions containing a common 3' terminus but differing in the extent of 5' flanking regulatory sequences, reveals that this complex regulatory element functions in a tissue specific manner, such that the enhancer activity predominates in lymphoid tissues, but not in non-lymphoid tissues.

In addition to tissue-specific regulation, MHC class I gene expression is known to be affected by immunomodulators, 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. Dr. Dinah Singer's laboratory has recently observed that the hormone TSH specifically reduces transcription of endogenous class I genes in cultured thymocytes. This decreased transcription is cAMP mediated and TSH receptor dependent. The ability of cAMP to reduce class I gene expression is not limited to the thymocyte line; a variety of cells including lymphoblastoid and fibroblasts also decrease class I expression in response to cAMP. A variety of other agents also have been shown to modulate class I expression. Interestingly, the well-characterized transcriptional activator, c-jun, has been shown by Dr. Singer's lab to be a negative regulator of class I expression (9285).

Dr. Dinah Singer's laboratory has identified a new MHC subregion, M, which contains a set of class I genes which are highly divergent from other members of the MHC family. The three most closely related members of the M family have been isolated and sequenced. All three are capable of encoding protein products, although no transcription has yet been observed. Transgenic mice have been generated in which the expression of one of the genes, M1, is directed by a viral LTR promoter. Analysis of the expression of M1 in these lines is in progress (9279).

Studies in Dr. Weissman's laboratory have focused on elucidating the genomic organization of the human ζ (zeta) gene, which codes for a T cell

antigen receptor subunit that is a key structure in receptor mediated signal transduction (9291). Studies have determined that the cDNA encoded for by zeta is encoded by at least 8 distinct exons that span at least 15kE of genomic DNA. Ribonuclease protection assays have revealed multiple sites of transcription initiation, consistent with the lack of a classic TATA or CAAT in the 5' region of the gene. There is a large first exon whose full size is yet to be determined. A variable number tandem repeat (VNTR) restriction fragment polymorphism exists within the ζ gene. This polymorphism has been localized to a 6KB BamHI fragment that encodes exons 4 through 6. Analysis of genomic DNA from different individuals indicates multiple alleles within the population. A linkage pattern for this VNTR is being established.

Dr. Weissman's laboratory has characterized a region at the 3' end of the ζ gene that is highly homologous on a nucleotide level to the murine η exon (9291). Despite the presence of this region in genomic DNA there is little evidence for a transcript containing this region spliced to ζ . However,

preliminary analysis of human T cells suggests that a species with characteristics similar to, but somewhat larger than, the murine η does in fact exist in human cells. This species has an Mr consistent with that predicted from the splice product. This species is present at low levels and its level appears to increase relative to ζ on activation of normal human PBL's. Studies are underway to determine whether this species is in fact the human η equivalent or whether it may in fact represent another alternative splice of the ζ gene.

5. LYMPHOCYTE EFFECTOR FUNCTION

Dr. Pierre Henkart's laboratory has sought to test the granule exocytosis model for lymphocyte cytotoxicity by examination of the cytotoxic activity of RBL cells transfected with genes for cytotoxic lymphocyte granule components (9251). RBL, a rat mucosal mast cell tumor line which degranulates in response to cross-linking its IgE Fc receptor is not cytotoxic but acquires 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 not as potent as cloned CTL. When target DNA degradation 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. Double and single RBL transfectants which express cytolysin and the granule serine protease granzyme A (gza) have been prepared. As expected, RBL-gza transfectants with protein expression levels comparable to cloned CTL show no cytotoxic activity. 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. When DNA breakdown in tumor targets was examined, it was observed with RBL-cy-gza effectors, although not as potently as with CTL effectors. These preliminary results support earlier evidence from Dr. Henkart's lab suggesting that granzyme A triggers target DNA breakdown after gaining access to the target cell cytoplasm. Drs. Segal and Wunderlich have shown that the growth of selected lines of

human tumor cells can be inhibited by peripheral blood T and K lymphocytes from normal human donors, when these PBL are targeted against the tumor cells with bispecific antibodies (9250, 9254). Bispecific antibodies, with specificities for triggering structures on cytotoxic cells and for cell-surface structures on target cells, redirect the target-cell specificities of cytotoxic cells. This antitumor activity has been demonstrated in vitro and also in vivo by using tumor neutralization (Winn) assays with immunodeficient mice. The inhibition of tumor growth, which is measured over a period of days, is mediated by mechanisms at least in part different from those responsible for tumor cell lysis that occurs within several hours. Two lines of evidence support this important difference. First, in the case of a colon carcinoma cell line, for example, the inhibition of tumor growth was mediated in vitro by γ -interferon and TNF- α , which were secreted by the targeted T and K lymphocytes. These cytokines do not lyse tumor cells within several hours. Second, these cytokines blocked not only the growth of tumor cells bound to the targeted cytotoxic cells, but also the growth of bystander tumor cells. The targeted PBL blocked the growth of bystander tumor cells in vitro and also in Winn assays in vivo. Lytic mechanisms do not harm bystander cells. These observations suggest that if tumor cells are near the triggered cytotoxic cells, they may not escape the antitumor activity simply by failing to express tumor antigens or by failing to be directly accessible to the targeted effector cells.

6. 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 (9275). In addition, Dr. Singer's laboratory demonstrated that rejection of skin allografts across a class II MHC barrier requires the production of endogenous IFN-gamma, presumably to induce class II expression on all the cells of the graft and make them recognizable by class II allospecific effector cells (9275). Finally, Dr. Singer's laboratory has assessed the cellular mechanisms mediating the rejection of fetal pancreas and Islet cell allografts (9275).

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' laboratory (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. 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. Veto 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 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. The mechanism by which veto cells suppress CTL responses has not been previously defined. Preliminary studies have shown 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. 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 appears to be due to both suppression of host T cell function and the presence of growth factors.

The elimination of cells expressing T cell surface markers from marrow is of interest both in allogeneic and autologous marrow transplantation -- as a means of preventing graft versus host disease in allogeneic marrow transplantation and as a means of eliminating or purging malignant cells expressing T cell surface markers from marrow in treating T cell neoplasms by autologous marrow transplantation. Dr. Gress' laboratory has developed approaches for depleting normal and malignant T cell marrow populations by using elutriation and deriving monoclonal antibodies specific for cell surface molecules unique to T cells (9288). These approaches have been used to develop clinical protocols to assess the feasibility of utilizing allogeneic HLA-mismatched, T cell depleted allogeneic marrow and autologous marrow purged of malignant T cells in the treatment of aggressive 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 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. 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.

Studies from Dr. Shearer's laboratory (9264) have focused on the effects of T lymphocyte function on treatment of mice with cyclosporin A (CsA). Exposure of mice in vivo with different doses of CsA indicate that lower doses of CsA selectively abrogated the MHC self-restricted T helper cell (Th) pathway of the allogenic response, but left the allo-restricted Th pathway functionally intact. In vitro studies in which human peripheral blood leukocyte (PBL) were stimulated with HLA alloantigens in the presence of different concentrations of CsA, suggested a similar differential sensitivity of human allo-specific Th pathways to CsA. Th analyses of renal allografted patients on immunosuppressive drugs indicated that a functionally intact self-restricted CD4⁺ Th pathway was associated with and predictive for kidney allograft rejection.

Other experiments from Dr. Shearer's laboratory (9260) indicate that murine cytomegalovirus (MCMV) infection can synergize with a graft-versus-host (GVH) reaction to make the GVH more severe. MCMV was found to provide a class II-like signal to synergize with a class I GVH, but does not appear to provide a class I-like signal and synergize with a class II GVH. The antiviral drug DHPG can reduce MCMV infection, but does not reduce the severity of GVH in MCMV-GVH synergy. Studies of this type may help to elucidate the synergistic effects of CMV infection and GVH disease in human bone marrow transplantation.

7. TUMOR IMMUNOLOGY

In Dr. Hodes' laboratory, analysis of TCR V β 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 V β 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 V β usage and T cell specificity. The response to syngeneic tumors in this system therefore

does not show any detectable predominance in TCR V β expression.

Animals are naturally endowed with cytotoxic effector cells that are part of the host's defenses against microorganisms and alien or rogue cells, including cancer. In humans, these cells are found among lymphocytes, macrophages or monocytes, and polymorphonuclear cells. Dr. Segal's laboratory has used bispecific antibodies to change cytotoxic effector cells, so that irrespective of their natural reactivity pattern, they will react against tumor cells and virally infected cells that are selected by the investigator. Bispecific antibodies, with specificities for triggering structures on cytotoxic cells and for cell-surface structures on target cells, redirect the target-cell specificities of cytotoxic cells. In order to generate bispecific antibodies that would be economical to produce and suitable for use in vivo, a project has been initiated to produce by genetic engineering, a single polypeptide chain with the antigen binding ability of a bispecific antibody (9289). The overall design is to link two different single chain Fv (sFv) constructs using a polypeptide spacer to join the C terminus of one sFv to the N terminus of the other. Several plasmids containing inserts encoding sFv molecules molecules have been produced. 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.

Drs. Segal and Wunderlich have demonstrated that targeted human PBL block the growth of human ovarian carcinoma cells established in the peritoneal cavity of immunodeficient mice (9250, 9254). In short term assays, where mice were sacrificed 11 days after treatment and peritoneal lavages were tested for tumor cells, it was 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. 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.

Drs. Segal and Wunderlich have initiated studies of targeted cytotoxicity in vivo, using a fully syngeneic mouse model consisting of mouse mammary tumors induced by the mammary tumor virus (MTV) (9250, 9254). High frequencies of C3H mice expressing the virus spontaneously develop mammary tumors, and a variety of murine mammary tumor lines have been established. Moreover, monoclonal antibodies against the major MTV envelope glycoprotein, gp52, have been obtained that bind selectively to tumor cell surfaces. Drs. Segal and Wunderlich 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 not at all to spleen cells. When crosslinked to an anti-murine CD3 mAb, the P2AE12 x anti-CD3 bispecific antibody induced murine T cells to lyse mammary tumor lines and spontaneous mammary tumors and blocked the growth of mammary tumor cells in culture. The mouse mammary tumor model therefore appears promising as a totally syngeneic murine system for studying immune targeting with bispecific antibodies in vivo.

8. FLOW CYTOMETRY

The EIB flow cytometry laboratory (9255) continues to support multiple investigations which involve quantitative, single cell, multiparameter analysis of cells prepared from a variety of species/tissues, as well as a spectrum of in vitro cultured cells (9275, 9268, 9273, 9265, 9258, 9266, 9259, 9257, 9287, 9288, 9290).

During the past year, the EIB flow cytometry laboratory (9255) has installed, and tested new flow cytometry instrumentation. A series of hardware modifications have been implemented and data acquisition and analysis software has been modified or developed in order to enhance functionality, flexibility, user-interface and throughput of flow cytometry instrumentation and software.

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

PROJECT NUMBER

Z01 CB 09250-26 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
 Delia Mezzananza Guest Researcher

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

NCI, NIH, Bethesda, Maryland 20892

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

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.)

Monomeric bispecific F(ab)'₂ antibodies were used to redirect the antigen specificity of T-lymphocytes from normal human donors, so that they became cytotoxic for an allogeneic ovarian carcinoma cell line. The altered lymphocytes killed the tumor cells in vitro and also blocked intraperitoneal growth of the tumor cells in immunodeficient mice, even though tumor growth was established prior to treatment. The antitumor effects in vivo were found both upon examining peritoneal lavage fluid 2 weeks after treatment and by monitoring host survival. Treatment of tumor-bearing mice, consisting of two injections spaced over four hours, doubled their mean survival time relative to controls.

Bacterial lipopolysaccharide (LPS), including an attenuated form, was used to nonspecifically activate mouse cytotoxic T lymphocytes (CTL), which then were retargeted with bispecific antibodies to lyse syngeneic tumor cells in vitro. The activation process involved CD8⁺ cytolytic precursor cells, CD4⁺ T-helper cells, and accessory cells that, by radiation sensitivity, appeared to be B lymphocytes. Cell mixing experiments, using splenocytes from LPS-responsive and nonresponsive, MHC-matched mouse strains showed that the accessory cells were the main cellular target for LPS.

Project Description

Major Findings:

The focus of this laboratory has been on antitumor cytotoxic effector cells and factors that influence their generation. This year we have extended our studies of a) retargeting the specificity of human T lymphocytes so that they react against human tumor cells and b) nonspecific activation of mouse cytotoxic T lymphocytes that can be retargeted to react against syngeneic tumors. In addition, we have evaluated mouse mammary adenocarcinomas induced by mammary tumor virus as a possible model for retargeting T cells in a fully syngeneic environment.

1. Retargeting human T lymphocytes against human tumor cells. The frequency of circulating human cytotoxic T lymphocytes (CTL) that react selectively with an autologous tumor is vanishingly small. However, other CTL can be redirected to react with a selected tumor, irrespective of their native antigen specificity. To this end, we have used bispecific antibodies that bind on one hand to triggering sites on the CTL, such as the T-cell antigen receptor complex, and on the other hand to cell-surface antigens on the tumor cells. Our bispecific antibodies consist of two covalently crosslinked murine monoclonal antibodies. The criteria for choosing an antitumor antibody include highly selective binding to the tumor cells in contrast to normal cells. Crosslinking of the T-cell triggering sites, which occurs when the bispecific antibodies bind effector to target cells, initiates cytotoxic activity by the effector cells. In previous studies carried out in vitro, we found that the antitumor activity of retargeted human T cells can be mediated by either of two different mechanisms. Antitumor effects can result from cytolysis, which is mediated by CD8⁺ T cells in our test systems and requires contact between the effector and tumor cells. Alternatively, antitumor effects can result from cytokines, such as tumor necrosis factor and gamma interferon, that are released by both CD8⁺ and CD4⁺ T cells in our test systems and inhibit the growth not only of bound tumor cells but also of neighboring tumor cells that are not in contact with the effector cells.

Earlier, we established that retargeted human T lymphocytes from normal donors are toxic for a human ovarian carcinoma cell line, OVCAR-3, both in vitro and in vivo. In the latter case, retargeted effector cells given intraperitoneally blocked the growth of ovarian carcinoma cells established 4-6 days earlier in the peritoneal cavity of immunodeficient athymic mice. About 60% of the treated mice had tumor-free peritoneal lavage fluid 2 weeks later, compared to about 20% of mice treated by a variety of control procedures.

This year, progress has been made in two areas of retargeting human T lymphocytes against ovarian carcinoma cells. First, we used a different form of bispecific antibodies against the T-cell receptor complex and ovarian carcinoma cells. Instead of bispecific antibodies crosslinked randomly through lysyl residues, we used monomeric bispecific F(ab)₂ antibodies crosslinked through the hinge sulfhydryl residues with a thioether linkage. This approach offers a variety of advantages over using bispecific antibodies in vivo. One advantage

is that the crosslinking process does not have the risk of inactivating the antibody combining sites. Moreover, the thioether linkage will not be reduced in vivo. Furthermore, the bispecific antibodies are monomeric, rather than mixed in size. Finally, the F(ab)'₂ fragments are less likely than aggregates of whole immunoglobulin to be cleared quickly in vivo or to mediate T-cell destruction.

We demonstrated that the monomeric bispecific F(ab)'₂ antibodies mediate retargeting of normal human T cells against the ovarian carcinoma cell line both in vitro in cytolytic assays and in vivo in short-term assays. The in vivo tests involved injecting immunodeficient mice intraperitoneally on day 0 and then treating them on day 4 with intraperitoneal injections of preactivated human peripheral blood lymphocytes and bispecific antibody. Preactivated effector cells were used, because in previous studies we found that the frequency of targetable peripheral blood T cells increases markedly, if the T cells are activated for several days in vitro by IL-2 and immobilized antibodies that crosslink the T-cell receptor complex. About two weeks after treatment, lavages of the peritoneal cavity were checked for tumor cells. About eighty percent of mice treated with PBL and the bispecific reagent were protected, compared to 6-20 percent among control groups.

The second advance has come from monitoring the effects of treatment on animal survival. The mean survival time was 104 days for tumor-bearing mice treated with preactivated PBL and bispecific F(ab)'₂ antibodies on day 4 after tumor injection. This was 3.5 times that of untreated mice and twice that of mice treated with lymphocytes alone or lymphocytes with either parental antibody alone. Two factors that limited the effectiveness of the treatment were a) giving it on only one day and b) the aggressiveness of the tumor. By day 4 after tumor injection, pancreatic invasion by tumor cells was detected in 5 out of 10 mice examined.

2. Nonspecific activation of mouse cytotoxic T lymphocytes. Two fundamental requirements of cytotoxic T cells are that they be sufficiently activated to produce and release the effector molecules that mediate cytotoxicity, and that they express receptors capable of triggering release of the cytotoxic molecules following ligand binding. Retargeting T cells with bispecific antibodies provides the triggering receptors that recognize the tumor cells of choice.

This year, we tested bacterial lipopolysaccharide (LPS) as a possible source of nonspecific CTL activation. LPS in small doses might be a natural activating factor in the lining of the gastrointestinal tract, and, particularly in an attenuated form, it might be useful for intentionally activating CTL in vivo. With in vitro cytolytic assays, we found that freshly explanted mouse spleen cells, which were retargeted, did not lyse syngeneic tumor target cells. The bispecific antibodies were against the T-cell receptor complex and the tumor cells. In contrast, splenocytes that were cultured for 5 days with ng/ml levels of native LPS or an attenuated form and were then retargeted, exhibited strong cytolytic activity. We analyzed the cellular basis for LPS-activation of the targetable effector cells by using cellular depletion and reconstitution tests. We found that the response used not only CD8⁺ cytolytic precursor cells but also

CD4⁺ T-helper cells and accessory cells. CD8⁺ T cells could provide helper-cell activity, but not as effectively as CD4⁺ T cells. The function of accessory cells was not affected by mitomycin C, but it was blocked by 3300R radiation and thus probably resulted from B lymphocytes. By mixing isolated classes of splenocytes from LPS responsive and nonresponsive, MHC-matched mouse strains, we determined that the accessory cells were the primary cells responding to LPS. As expected, the response was blocked by antibodies against IL-2 or the high-affinity IL-2 receptor. Thus, LPS was able to activate CTL by a multicellular process very similar to that involved with antigen-specific CTL responses.

3. Evaluation of mouse adenocarcinomas induced by mammary tumor virus (MTV) as a possible syngeneic model for retargeting CTL. Greater than 70% of breeding C3H mice, weaned by MTV⁺ mothers, develop spontaneous mammary adenocarcinomas by one year of age, and many of these mice develop metastases. The tumor cells express the viral envelope glycoprotein gp52, against which a variety of monoclonal antibodies are available. Also, tumor lines that express gp52, with or without associated virus production, are available. In preliminary studies, we found by flow cytometry that a monoclonal antibody against MTV gp52 bound to MTV⁺ tumor lines and to freshly explanted MTV-induced tumor cells but poorly if at all to splenocytes from MTV⁺ tumor-bearing mice. Moreover, mouse splenic T cells, retargeted to react with gp52, blocked the growth of a syngeneic gp52⁺ tumor cell line and lysed both the tumor cell line and freshly explanted syngeneic tumor cells in vitro. Freshly explanted splenocytes were not lysed. Thus, MTV-induced mouse adenocarcinomas may prove to be an appropriate syngeneic model for targeting T cells against tumors.

Proposed Course

We anticipate that next year our work will focus on nonspecifically activating murine cytotoxic antitumor cells and on retargeting mouse cytotoxic cells toward selected syngeneic tumor cells.

1. Nonspecific activation of mouse CTL. We plan to test the ability of bacterial LPS, including an attenuated form, to activate CTL in vivo and to continue our analysis of how LPS activates CTL. The cellular target of LPS in this situation appears to be B lymphocytes, which serve as the primary accessory cells in a multicellular process. We plan to try to identify the B lymphocyte functions that mediate the LPS response; for example, we will look at the role of costimulatory factors, such as selected adhesion molecules. In previous studies, we observed that LPS and selected macromolecular polyanions activated cytotoxic antitumor NK cells over a 5-day period by a multicellular process very similar to that involved with LPS activation of CTL. In this response, too, B lymphocytes were the primary target of LPS. We also found that the level of activation of NK cells by LPS and polyanions in vitro is strain-dependent. We want to determine whether there are also strain-dependent differences in the activation of CTL by LPS and whether, in both models, the strain-dependent differences rest with B lymphocytes. If B lymphocytes are the source of the strain differences, we will also try to identify the B-lymphocyte functions that are responsible.

2. Retargeting mouse cytotoxic cells toward syngeneic tumor cells. We intend to extend our analysis of MTV-induced mouse tumors as an animal model for retargeting CTL against syngeneic tumors, both in vitro and in vivo. We plan to determine whether retargeted CTL can block MTV-induced tumor growth in vivo.

Initially, this issue will be tested by tumor neutralization in Winn-type assays. We intend, however, to progress quickly to working with established tumors, including primary tumors. We want to determine if retargeted CTL given intravenously can home to tumor sites. We want to assess the effects of cell-free tumor antigen, gp52, on antitumor effector cell activity, and whether there is expression of gp52 by some normal cells that will react with retargeted CTL. We will look for differences in the retargeting effectiveness of various forms of bispecific antibodies, particularly those that are randomly crosslinked through lysyl residues, monomeric bispecific antibodies that are produced by quadromas, and genetically engineered single chain bispecific antibodies. The consequences of CTL activation on the effectiveness of retargeting in vivo will be examined, including activation resulting from the bispecific reagent itself, anti-CD3, and bacterial LPS. We will draw on the availability of MTV-induced tumor lines that express gp52 with or without concomitant virus production and also on MHC-matched mouse strains that are MTV⁺ or MTV⁻.

Publications:

Wunderlich JR, Shearer GM. CTL Function. In Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM and Strober W. Current Protocols in Immunology, Green Publishing Associates, New York, Volume 1, 1990. p. 3.11.1.

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. *Molecular Immunology*. 1990;27:1339-1342.

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.

Wunderlich JR, Hodes RJ. Principles of tumor immunity: biology of cellular immune responses. In DeVita Jr VT, Hellman S, Rosenberg SA, *Biologic therapy of cancer: principles and practice*. Lippincott, Philadelphia. In Press.

Segal DM, Qian J-h, Andrew SM, Titus JA, Mezzanzanica D, Garrido MA, Wunderlich JR. Cytokine release by PBL targeted with bispecific antibodies and its role in blocking tumor growth. *Annal N.Y.Acad. Sci*. In Press.

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*. In Press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09251-21 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	S. Winslow	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:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- | | | |
|---|--|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors | | |
| <input type="checkbox"/> (a2) Interviews | | |

B

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

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 acquires a potent lytic activity against IgE coated red cells when transfected with the mouse cytolysin (cy) gene. This potency is equivalent to that of cloned CTL with this target. We have found that electroporation gives much higher (2 orders of magnitude) transfection efficiency than the calcium phosphate technique originally used. Furthermore the cloned RBL electroporation transfectants (RBL-cy) give even more potent lytic activity against RBC targets than did those generated with calcium phosphate. Using tumor target cells, the RBL-cy give good cytotoxicity, although not as potent as cloned CTL. When target DNA degradation 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). As expected, RBL-gza transfectants with protein expression levels comparable to cloned CTL show no cytotoxic activity. 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. 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.

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 acquires a potent lytic activity against IgE coated red cells when transfected with the mouse cytolysin (cy) gene. This potency is equivalent to that of cloned CTL with this target. We have found that electroporation gives much higher (2 orders of magnitude) transfection efficiency than the calcium phosphate technique originally used. Furthermore the cloned RBL electroporation transfectants (RBL-cy) give even more potent lytic activity against RBC targets than did those generated with calcium phosphate. Using tumor target cells, the RBL-cy give good cytotoxicity, although not as potent as cloned CTL. When target DNA degradation 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). As expected, RBL-gza transfectants with protein expression levels comparable to cloned CTL show no cytotoxic activity. 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. 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.

Proposed course:

We need more clones of double transfectants expressing both cytolysin and granzyme A to be sure that the DNA breakdown observed is not a statistical fluke. We plan on trying various manipulations of this system to improve the efficiency of tumor cell lysis by the RBL transfectants. The first one, currently in progress, is to co-transfect genes for the CTL granzyme B along with the cytolysin and granzyme A genes in all combinations. We have also been exploring the use of butyrate to enhance the expression of granules in RBL cells. In collaboration with Michael Curran, we have found conditions under which butyrate appears to enhance the expression of granules morphologically. We will culture the transfectants under these conditions to see if the lytic activity is improved.

Publications:

Shiver JW, Penkart HA. A Noncytotoxic mast cell tumor line exhibits potent IgE-dependent cytotoxicity after transfection with the cytolysin/perforin gene. Cell 1990;62;1174.

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

PROJECT NUMBER

Z01 CB 09254-17 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

SUMMARY OF WORK (Use standard unrounded 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 ⁵¹Cr 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.

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 ⁵¹Cr-release over 4 hr, in vitro tumor growth inhibition 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 reproducible

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)

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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 bispecific antibodies, and its role in blocking tumor growth. *Annal N.Y. Acad. Sci.* in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09255-16 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:

3.0

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The 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.

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 V β 8 and expression of potentially autoreactive T cell receptors. Unlike CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ thymocytes which share these characteristics, Ly-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-6C⁺ thymocytes progress through the CD4/CD8 developmental pathway in an ordered sequence identical to that of conventional Ly-6C⁻ thymocytes. Taken together, these results suggest that Ly-6C⁺ thymocytes are the precursors of CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ thymocytes, and that Ly-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 conventionally 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 F₁ mice. Analysis of host and donor populations during both the acute immunosuppressive 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 V β gene products, b) analysis of negative selection of the T cell repertoire, and c) analysis of ligands mediating V β -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 V β 11, V β 12, and V β 5. In

other studies the $V\beta$ 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 $V\beta$ gene products. While restricted $V\beta$ 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 by sample input, and not by interaction with computer software.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09257-16 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanisms of Cellular Immune Responses

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

PI:	S. Shaw	Section Chief	EIB, NCI
Others:	G. Ginther-Luce	Chemist	EIB, NCI
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COOPERATING UNITS (if any)

Otsuka Pharmaceuticals: Walter Newman

LAB/BRANCH

Experimental Immunology Branch

SECTION

Human Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

7.0

PROFESSIONAL:

7.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

Our studies emphasize two fundamental areas: 1) characterizing 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. Progress has been made in understanding the molecular basis of T cell interactions with endothelium. Two new molecular pathways are being elucidated. A subset of memory T cells bind to the inducible ligand ELAM-1 on endothelial cells; since this pathway does not require prior T cell activation, it may be of primary importance in the initial attachment of memory T cells to inflamed endothelium in vivo. We have defined many characteristics of the molecule CD31 on a subset of T cells which make it a very attractive candidate for regulating T cell adhesion to endothelium. Not only does CD31 mediate adhesion, but it also powerfully induces adhesion by the multiple integrins which are present but relatively nonadhesive on resting T cells. T cell interaction with endothelium via three other molecular pathways: VLA-4/VCAM-1, LFA-1/ICAM-1 and LFA-1/ICAM-2 has also been systematically analyzed. Not only CD3 and CD31, but also the T cell surface molecules CD7 and CD28 can augment the adhesive function of multiple integrins expressed by T cells. Furthermore, adhesion molecules regulate T cell activation, as illustrated by our recent studies with VLA-4/VCAM-1, which confirm and extend ongoing studies of LFA-1/ICAM-1 interactions. Detailed analysis of phenotypic heterogeneity among peripheral blood CD4+ T cells, and more recently CD8+ T cells, identifies marked complexity of regulation of surface phenotype such as: 1) CD4 memory cells can be subdivided into two subsets based on quantitative differences in expression of the CD45RB isoform; 2) Memory cells seem to be much less abundant among CD8 cells than CD4 cells; 3) VLA-4 stands out as an important parameter of differentiation both among CD4 cells and among CD8 cells. In short, our studies highlight and elucidate the relationships between adhesion, activation and differentiation.

Project Description

Major Findings

Our interests are in two broad areas: accessory/adhesion molecules which facilitate T cell recognition; and human T cell subsets. This dual interest arose from our previous findings that adhesion molecules were differentially expressed on T cell subsets. The concept that adhesion molecules often mark T cell subsets has been confirmed and extended by many aspects of our studies this year, including analysis of $\beta 1$ integrins, CD31 and the ELAM-1 ligand.

Most of our analysis continues to be done with subsets of "fresh" resting human peripheral blood T cells. We have emphasized studies of such normal "unperturbed" cells because we have learned how rapidly and dramatically phenotype and function change with culture of cells. Rigorous purification has been done by immunomagnetic negative selection to minimize perturbation of the cells during the process of purification. Most of our past and present studies have emphasized CD4+ T cells, but the wealth of new insights emerging from CD4+ cells have prompted us to extend some of the analysis to CD8+ cells this year.

One context in which adhesion is critically important is T cell binding to endothelium, which is critical to lymphocyte recirculation and influx into sites of inflammation. With the benefit of an excellent collaboration with Walter Newman at Otsuka America, we have systematically analyzed the role of four receptor/ligand interactions that mediate adhesion of peripheral human CD4+ T cells to cultured human umbilical vein endothelial cells (HUVEC): T cell LFA-1 binding to ICAM-1 and an alternative ligand (presumably ICAM-2), T cell VLA-4 binding to VCAM-1, and T cell binding to ELAM-1. Some of the most novel observations have emerged regarding ELAM-1, a rapidly inducible endothelial cell surface adhesion molecule, which was previously thought to mediate endothelial cell binding for granulocytes but not T cells. We demonstrated that ELAM-1 contributes to the binding of resting CD4+ T cells to IL-1-induced human endothelial cells. Detailed studies with purified ELAM-1 immobilized on plastic reveal two unique features of T cell adhesion to purified ELAM-1. First, ELAM-1 exclusively mediates adhesion of memory T cells. Second, memory T cell binding to ELAM-1 is completely independent of acute activation events that regulate integrin-mediated adhesion. Thus, ELAM-1 may be of primary importance in the initial attachment of memory T cells to inflamed endothelium in vivo and to the preferential migration of memory T cells into tissue and inflammatory sites.

The findings on ELAM-1 are best understood in the context of our systematic comparisons between it and the three other defined pathways of T cell/endothelial interactions. Contributions of these four pathways depend on the activation state of both the T cell and HUVEC, and the differentiation state of the T cell. ELAM-1 plays a significant role in mediating adhesion of resting CD4+ T cells to activated HUVEC. LFA-1 adhesion dominates with PMA-activated T cells but the strength and the predominant LFA-1 ligand is determined by the activation state of the HUVEC; while ICAM-1 is the dominant ligand on IL-1-induced HUVEC, another ligand (presumably ICAM-2) dominates

binding to uninduced HUVEC. Adhesion via VLA-4 depends on induction of its ligand VCAM-1 on activated HUVEC; PMA-activation of T cells augments VLA-4-mediated adhesion, both in the model of T/HUVEC binding and in a simplified model of T cell adhesion to VCAM-1-transfected L cells. Unlike LFA-1 and VLA-4, ELAM-1-mediated adhesion is not increased by T cell activation. Differential expression of adhesion molecules on CD4+ T cell subsets understood to be naive and memory cells also regulates T/HUVEC adhesion. Naive T cell adhesion to HUVEC is mediated predominantly by LFA-1 with little or no involvement of the VLA-4 and ELAM-1 pathways. In contrast, memory T cells bind better to HUVEC and utilize all four pathways. These studies demonstrate that the dominance/hierarchy of these four pathways varies dramatically with the activation state of the interacting cells and the differentiation state of the T cell.

In the course of our studies of markers of T cell subsets, we have begun to investigate another molecule, CD31, which we now hypothesize is involved in another very important pathway of T cell interaction with endothelium. Many of the known adhesion molecules on T cells are differentially expressed on subsets of T cells; therefore, we consider any marker of T cell subsets as a candidate for an adhesion molecule. Over the past several years we have screened more than 400 established monoclonal antibodies (specific for both known and poorly defined molecules) for detection of heterogeneity among human peripheral blood T cells. More than 10 new kinds of heterogeneity were observed, and all those markers are now being investigated. Our investigation of CD31 is the one which has progressed the most rapidly and dramatically. We observed a characteristic bimodal distribution of CD31 on peripheral blood lymphocytes. CD31, which is also known as PECAM-1/endoCAM, had been inferred by others to mediate adhesion between leukocytes/platelets and endothelial cells and therefore potentially function in immunity, inflammation and wound healing.

We have elucidated four novel features of CD31 on T lymphocytes: 1) Unique subsets of CD4+ and CD8+ T cells, including all CD8 naive cells, express CD31; 2) Purified CD31 is sufficient to mediate T cell adhesion; this is direct evidence that CD31 protein per se is an adhesion ligand. The inability of CD4 memory cells, which are primarily CD31-negative, to bind to CD31 is consistent with, but does not prove, the interpretation that purified CD31 may engage in homophilic interaction with T cell CD31. 3) Engagement of CD31 powerfully induces the adhesive function of $\beta 1$ and $\beta 2$ integrins, particularly among naive T cells. In last year's annual report, we described studies showing that multiple integrins in resting T cells were functionally inactive until induced to assume adhesive function by CD3/TCR crosslinking, or by pharmacologic agents. CD31, like CD3, is a powerful inducer of integrin adhesion. We believe that CD31 is a critical early participant in a coordinated series of receptor/ligand interactions or "adhesion cascade" that results in T cell adhesion. CD31 binding to its ligand mediates some adhesion of resting T cells; probably more importantly, it is a powerful inducer of integrin adhesion. 4) CD31 preferentially induces binding to VCAM-1, fibronectin and laminin which are particularly important in T cell capture by endothelium and subsequent migration; in contrast, crosslinking of the CD3/T cell receptor

preferentially induces binding to ICAM-1, which is of paramount importance in antigen-specific recognition. An important implication of this preferential adhesion induction is that there must be more than one biochemical mechanism for integrin regulation; it remains to be seen if the selectivity reflects involvement of $\beta 2$ and $\beta 1$ chains per se or is based on some other structural motif. Thus, CD31 functions in an "adhesion cascade" by inducing integrin-mediated adhesion of selected T cell subsets. CD31 may be particularly important in T cell interactions with endothelium, especially in high endothelial venules (HEV) of lymph nodes through which naive cells recirculate.

The foregoing studies of adhesion induction by CD31 fit with the concept that T cells must continuously and rapidly regulate their adhesive capacity. We are seeking other molecules, in addition to CD3 and CD31, which are able to regulate T cell adhesion. We find that antibody crosslinking of the CD7 and CD28 molecules results in the rapid induction of integrin-mediated adhesion to fibronectin and ICAM-1; CD7 is preferentially expressed on naive T cells and is unique in being a potent inducer of naive T cell adhesion. We propose that engagement of CD7 and/or CD28 by their natural ligands enhances integrin-mediated adhesion not only *concurrent* with TCR-occupancy, but also *preceding* TCR-occupancy to facilitate the cell contacts that are conducive to establishing TCR-occupancy and subsequent activation.

It has become apparent over the last several years from our work and that of others that adhesion molecules are not simply glue, but rather are often signal-transducing molecules. Previously, we have elucidated roles primarily for LFA-1/ICAM-1 and to a lesser extent CD2/LFA-3 in T cell activation. We are continuing to extend our understanding of those pathways, as well as others. Proliferative responses of resting T-cells generally require not only cross-linking of the T-cell receptor but also costimulatory signals from accessory molecules. Since three-cell systems provide TcR cross-linking at a site spatially "remote" from costimulation, they help distinguish adhesion from signal transduction; but the molecules that mediate costimulation in such systems have not been identified. We exploited a "three-cell" model consisting of: 1) resting human CD4+ T-cells as responders; 2) CD3 mAb OKT3 on latex beads as surrogate stimulators; 3) autologous monocytes as source of costimulation. T-cell proliferation in this system is observed with paraformaldehyde-fixed monocytes if they have been activated and IL-1/IL-6 is supplied. Our studies now demonstrate that costimulation by the monocytes is dependent on each of two receptor/ligand pathways CD2/LFA-3 and LFA-1/ICAM-1 since it is inhibited by each relevant mAb but not a variety of control mAb. The monocytes' transition from non-functional to functional accessory cell may reflect enhancement of the LFA-1/ICAM-1 pathway, since cell surface expression of ICAM-1 on stimulated monocytes is markedly increased (3-4 fold), but LFA-3 is not. The hypotheses that CD2 and LFA-1 could each mediate costimulation was tested in simplified model systems in which the monocyte was replaced with immobilized CD2 mAbs or purified ICAM-1 presented on a separate surface from the CD3 mAb. The results in these simplified models demonstrate that on resting T-cells either CD2 or LFA-1 molecules alone can mediate "remote" costimulation. Costimulation requires IL-1/IL-6 both in

the weaker LFA-1-ligand mediated costimulation and at lower CD2 mAb concentrations in the stronger CD2 mAb-mediated costimulation. Thus: 1) the accessory cell function of stimulated fixed monocytes in T-cell proliferation requires both the LFA-1/ICAM-1 and CD2/LFA-3 pathways; and 2) the T-cell molecules CD2 and LFA-1 can give costimulatory signals that can act in a "remote" fashion.

Although initial investigation of T cell interaction with endothelial cells was limited to adhesive interactions, we now are investigating the molecules in T/endothelial adhesion for their role in facilitating T cell activation. Of particular importance is the investigation of two new molecular pathways involved: T cell VLA-4 binding to endothelial VCAM-1 and an unknown T cell ligand binding to endothelial ELAM-1. Underlying this investigation was 1) the belief that endothelial cells may function as antigen-presenting cells in vivo; 2) the knowledge that one of the molecules binding studied, VCAM-1, is expressed on many "professional" APCs. We observed that VLA-4/VCAM-1 interactions provide a very powerful costimulus for T cell activation in two model systems. The first model system is the now "conventional" system of co-immobilization with CD3 mAb which by itself is not stimulatory. The second is a novel biochemically-defined model system which we developed as a more physiologic one: immobilized MHC class II plus the superantigen SEA. Resting T cells proliferate in either model system only if a costimulatory ligand is also present, and both VCAM-1 and ICAM-1 are cell surface ligands which mediate this function when biochemically purified. Similar requirements are observed for induction of secretion of the cytokines IL2, IL4 and GM-CSF. An extremely important "negative" result is the finding that immobilized ELAM-1 does not provide costimulation despite its ability to mediate adhesion. This provides strong evidence that adhesion per se is not the mediator of this costimulation, but that specific signaling events are occurring. Analysis of Ca⁺⁺ flux and inositol phosphate metabolism are underway to help elucidate the biochemical mechanisms of costimulation.

We have previously contributed to the concept that naive vs memory is a fundamentally important distinction among peripheral blood T cells and that isoforms of the CD45 molecule (CD45RA and CD45RO) are excellent markers of these distinct differentiation states. We have extended this analysis to another isoform of CD45: CD45RB. Two subpopulations can be identified within CD45RA⁻CD45RO⁺ cells; one population is CD45RB^{bright} and the other is CD45RB^{intermediate}. This contrasts with CD45RA⁺ naive cells which are uniformly CD45RB^{bright}. Since both subsets of CD45RA⁻ cells proliferate in response to recall antigen, we designate them MEM 1 (CD45RO⁺ RB^{bright}) and MEM 2 (CD45RO⁺ RB^{intermediate}). Both MEM 1 and MEM 2 subsets are seen in peripheral CD4⁺ T cells and also in secondary lymphoid organs (spleen, tonsil and lymph node). CD45RA and CD45RB expression are regulated independently during in vitro activation of naive cells; naive-phenotype cells when activated generally acquire a MEM 1 phenotype though with prolonged culture many acquire MEM 2 phenotype. When MEM 1 cells are activated (particularly with PHA and PMA) they tend to downregulate CD45RB expression whereas MEM 2 cells when activated (especially with SEA) tend to upregulate CD45RB

expression. In contrast to the stability of the CD45RA⁻CD45RO⁺ phenotype, the MEM 1 and MEM 2 phenotypes are relatively labile and may interconvert in vivo.

Major subsets of CD4⁺ T cells are defined by differential expression of the CD45 isoforms CD45RA/CD45RO, and the VLA β 1 chain (CD29). Although most circulating CD4⁺ T cells are CD45RO-CD29- ("naive") or CD45RO+CD29+ ("memory"), some CD45RO+CD29- and CD45RO-CD29+ cells also occur. Three color FACS analysis was used to define the surface phenotype of these 4 subsets, particularly with respect to VLA chains that associate with CD29. We suggest a model in which CD45RO-CD29- cells, which are most homogeneous and express low levels of most adhesion molecules, represent naive cells. The CD45RA to CD45RO isoform switch and the increase in CD29 expression are distinct maturation events which do not necessarily occur together. Acquisition of CD45RO is accompanied by changes in CD45RB expression. While increased CD29 expression is accompanied by increased expression of the associated VLA-5 and VLA-6 chains, VLA-4 can appear without increased CD29 on cells that express CD45RO. Adhesion molecules such as CD2, LFA-1 and LFA-3 undergo increase with either CD29 or CD45RO acquisition, but are expressed at highest levels on cells which have both CD29 and CD45RO. In addition, other markers are being identified which show unique patterns on these subsets, and promise to identify new adhesion molecules with new patterns of regulation. These findings extend the evidence regarding finely controlled regulation of adhesion molecules with T cell differentiation.

Differences between CD8 and CD4 T cells in their functional capacities and in tissue localization may be determined by surface phenotypic differences other than expression of CD8 vs CD4. We have systematically compared expression of many molecules on CD4 and CD8 T cells present in circulation and in secondary lymphoid tissues. There are strikingly fewer CD45RO+ cells (and reciprocal higher frequency of CD45RA+ cells) among CD8+ cells when compared to CD4+ cells; this pattern is observed in peripheral T cells (typically 90% of CD8's CD45RA but 50% of CD4's CD45RA+) or in spleen, tonsil, or lymph node (typically >70% vs <30% CD45RA+). Since many previous studies indicate that CD45RA+ T cells are naive and CD45RO+ are memory cells, these data suggest that a much smaller fraction of adult CD8+ cells have undergone antigen-specific priming than CD4+ cells. Other molecules also differ in expression on CD8 vs CD4 cells. Some appear to be secondary to the shift towards more naive cells among CD8 cells. Others, particularly VLA-4 (CD49d), do not fit the definition of naive cell markers, and their expression must reflect preferential utilization of this adhesion molecule by CD8 cells. The relatively naive composition of CD8 cells and their preferential expression of molecules like VLA-4 must contribute to differences in their functional capacities.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09258-13 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Immune Response Gene Regulations of the Immune Response In Vitro

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

PI: R. J. Hodes Section Chief EIB, NCI

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SECTION

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TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

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

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

In order to analyze the genetic regulation of T cell responses to NASE, a series of cloned lines were generated in BALB/c (H-2^d) as well as (H-2^b x H-2^a)F₁ T cells. Individual clones were restricted to recognizing NASE in the context of either A_αA_β or E_αE_β products. The antigen fine specificity of cloned NASE-specific T cells was also probed through the use of mutant NASE molecules and synthetic peptides. A consistent correlation was found between the fine specificity of a given clone and its MHC restriction specificity. A_α^bA_β^b restricted clones were selectively responsive to peptide 91-110; E_α^kE_β^k restricted clones were responsive to peptide 81-100.

The nature of the immunogenic peptides which are processed and presented to T cells was further evaluated using a panel of variant NASE peptides. It was observed that negative (inhibitory) interactions appear to occur between amino acids in peptides which interfere with T cell responses. Single amino acid changes, by eliminating such apparent negative interactions, result in the restoration of T cell stimulatory ability in these peptides.

The T cell receptors (TCR) expressed by NASE specific clones have been analyzed to determine the relationship between antigenic fine specificity and TCR expression. Preferential expression of specific V_α and V_β products was observed, e.g. expression of V_β4 by six of seven independent clones specific for NASE 91-110 in association with A_α^pA_β^p, and expression of V_β10 in five of nine independent clones specific for NASE 81-100 in association with E_α^kE_β^k. Conservation of receptor usage was also reflected in sequence analysis by PCR amplification of α and β chain cDNA.

Project Description

Major Findings:

1) Fine specificity and MHC restriction of T cell responses to NASE.

Monoclonal Th cell populations specific for NASE have been generated in BALB/c (H-2^d) and (B10xB10.A)F₁ (H-2^bxH-2^a) genotypes. Of the BALB/c clones tested, individual clones show MHC restriction for either I-A or I-E products. Individual (B10 x B10.A)F₁ clones respond to native NASE in association with either Aα^bAβ^b or Eα^kEβ^k. When the specificity of these clones was analyzed employing a series of overlapping 20 amino acid synthetic peptides corresponding to the NASE sequence, it was found that Aα^bAβ^b restricted clones were highly responsive to peptide 91-110; and not to other synthetic NASE peptides. In contrast, Eα^kEβ^k restricted clones were consistently responsive to peptide 81-100 and not to 91-110. These findings demonstrate that in T cell recognition of a complex and highly foreign protein antigen, a limited number of peptide epitopes are preferentially recognized by T cells in association with a given Ia molecule.

More detailed analysis of T cell fine specificity in response to NASE peptides was carried out using variants in which a single amino acid was altered from its native or wild type form. This analysis revealed that clones with specificity for the same native peptide and self MHC determinant may vary significantly in their fine specificity as assessed by responses to variant peptides. It was also observed that certain Eα^kEβ^k restricted clones responded to peptide 91-100 but not to 91-105. On the basis of this finding, it was suggested that interactions between amino acids in the 101-105 region with amino acids in the stimulatory 91-100 region were responsible for impaired ability to stimulate T cells. Consistent with this hypothesis, it was found that single amino acid changes including those in the 91-100 as well as those in the 101-105 region were capable of restoring stimulatory ability. Such changes appear to have their effect by eliminating inhibitory interactions among amino acid residues in the 91-105 peptide.

2) TCR expression and specificity of T cell recognition.

TCR Vα and Vβ expression of NASE-specific T cell clones was first analyzed by flow cytometry and mRNA analysis. Preferential expression of specific Vα and Vβ products was observed, including the expression of Vβ4 by six of seven independent clones specific for NASE 91-110 in association with Aα^bAβ^b, and expression of Vβ10 in five of nine independent clones specific for NASE 81-100 in association with Eα^kEβ^k. Vα1 was expressed together with Vβ10 in four of the latter five clones. Preliminary analysis of α and β chain expression has been carried out by PCR amplification of cDNA from these clones and sequencing in collaboration with Hans Ulrich Weltzien (Freiburg, FRG). This has revealed a restricted usage of J regions as well as striking sequence conservation in the receptors expressed by NASE-specific clones.

Proposed Course of Project:

Cloned T cell populations and synthetic peptides will be employed to further analyze the structural relationship between antigen fine specificity and TCR expression. Variant peptides will be used to more definitively characterize fine specificity differences between clones. Additional sequencing of TCR cDNA will be performed to define the correlation between specificity and receptor structure.

Publications: None.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09259-13 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

B

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

Allogeneic transplantation of bone marrow and lymph node cells matched to the host at major histocompatibility loci, but disparate at minor loci, can generate a severe graft-vs-host disease (GVHD). In Mls disparate transplants, selective expansion of elements of the splenic T cell receptor $V\beta$ repertoire indicated that Mls reactivity might play an important role in early GVHD. After transplantation of BALB/c (H-2^d, Mls^c) cells into irradiated DBA/2 (H-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 on 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. 90% of BALB/c--->DBA/2 mice died at 3-5 weeks after undepleted transplants, but >70% survived at 12 weeks after $V\beta$ depleted transplants.

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 $V\beta_{8.1}$. 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.

The mechanism(s) responsible from immune protection of mice to the intracellular parasite *Toxoplasma gondii* was studied. Immunization with a temperature sensitive mutant resulted in priming such that CD4+ and CD8+ spleen cells responded in vitro to *T. gondii* antigens by IL-2 and INF- γ production and CD8+ cells generated antigen-specific H-2 restricted cytotoxic T lymphocytes. CD8+ but not CD4+ T cells were crucial for protective immunity in immunized mice. Chronic *T. gondii* infection in Fl mice preceded the induction of chronic or acute parent-into-Fl GVHR. Acute GVHR resulted in *T. gondii* brain cysts and death, whereas chronic GVHR mice survived the infection.

Project Description

Major Findings1.) Analysis of T cell receptor V β repertoire in GVH.

Recent analyses of the T cell receptor repertoire have demonstrated that certain V β families are associated with the responses to Class II I-E and Mls antigens. T cells expressing certain V β markers predominated in the in vitro response to the relevant antigens and appear to be clonally deleted during thymic maturation in mice expressing these antigens. Because the graft-vs-host-reaction (GVHR) involves the response of donor lymphocytes to alloantigens on host cells, the V β expression of donor populations was investigated in GVHR strain combination selected because of Mls^a or class II I-E disparities.

This research had two objectives. The first was to determine the feasibility of reducing or preventing GVHR following bone marrow transplantation by removing only the T cells expressing host-reactive T cell receptors from the donor inoculum. The remaining donor T cells could then aid engraftment and provide disease resistance. The second was to investigate the changes in frequency and immune responsiveness of the host reactive cells during the course of GVHR. Exposure to a superantigen such as Staphylococcal enterotoxin B has been reported to produce an expansion of reactive cells, followed by a selective death and anergy of these cells. Experiments tracking host-reactive donor populations during GVHR were performed to determine whether the alloreactive cells in GVHR similarly followed a pattern of expansion, followed by selective cell death and/or anergy.

It was previously established that, during the GVHR, donor T cells expressing V β markers associated with responses to host alloantigens dominate the donor population. To investigate the role of such T cells in the induction of GVHR the host-reactive V β populations were depleted from the donor inoculum by the use of monoclonal antibodies against V β 6 and 8.1,2 (RR4-7 and KJ16) and anti-Ig coated magnetic beads. This treatment prevented the expansion of these T cell subpopulations following bone marrow transplantation in the BALB/c--->DBA/2 combination: V β 6⁺ cells constituted 55% of CD4 cells from mice receiving non-depleted donor lymph node (plus bone marrow) inocula, but less than 1% of CD4 cells from mice injected with V β depleted donor lymph node cells. Selective V β depletion significantly reduced acute GVHR mortality: 17/18 mice receiving non-depleted donor lymphocytes died by 5 weeks after marrow transplant, whereas 13/20 of the mice receiving V β depleted lymphocytes still survived at 15 weeks.

Selective V β depletion did not, however, completely eliminate GVHR-induced immune deficiency. DBA/2 host mice receiving V β 6 & 8.1,2-depleted BALB/c lymphocytes developed the early physical symptoms of GVHD -- weight loss, edema, and diarrhea. At 7 weeks, both B and T cell responses to mitogens were reduced compared with cell responses from host mice receiving only T depleted bone marrow (no lymphocytes); in co-cultures, cells from V β -

depleted transplants suppressed the responses of cells from T-depleted transplants. The presence of suppressive activity is common in early GVHR. At 17 weeks after marrow transplant, responses to allogeneic stimulators were variable; some V β -depleted transplant mice generated cytotoxic T cells and produced IL-2 to a degree comparable to that of untreated mice or to mice receiving only T depleted bone marrow transplants whereas others had lower responses. All mice receiving V β depleted lymphocytes had fewer T cells than mice receiving T depleted grafts. Thus, some degree of GVHR, even if transient, remained in the host mice receiving donor lymphocytes depleted of host reactive V β populations.

Two factors may have contributed to the persistence of GVHR in mice receiving V β depleted grafts. First, T cells expressing other V β chains may contribute to the response to Mls^a disparate stimulation. In normal BALB/c mice and in DBA/2 mice receiving BALB/c marrow and lymphocyte grafts, V β 9 expressing cells accounted for less than 2% of the CD population. In DBA/2 hosts receiving V β 6 & 8.1,2 depleted grafts, the V β 9 expressing cells accounted for 20% of the CD4 population. Second, BALB/c and DBA/2 mice differ at many loci, not simply Mls, and T cells responding to these disparities would not necessarily be eliminated by V β 6 & 8.1,2 depletion.

The second objective of this project was to investigate modulation of the numbers and responsiveness of host-reactive T cell populations during the course of acute GVHR. Two separate models of GVHR are being examined. The first model, as noted above, involves the induction of GVHR by transplantation of MHC matched, Mls mismatched donor lymphocytes and marrow into irradiated hosts (BALB/c-->DBA/2). During the first week the GVHR, the donor V β population expanded from approximately 10% of the CD4+ T cell repertoire to 60%. During the second week, however, the frequency of V β 6 cells decreased to 30% of the CD4 population. Further analysis was complicated by increasing morbidity in GVHR mice at 3-4 weeks. In the second model, C57BL/6 (B6) spleen cells were injected into unirradiated (C57BL/6 x B10.A(5R)F1) hosts. The donor cells react against the I-E^k and H-2D^d antigens of the host to produce an acute suppressive GVHR. While severe immune deficiency is observed in this model, little morbidity is found. Furthermore, by using congenic B6-Thy 1.1 mice as donors, the donor populations can be distinguished from the Thy 1.2+ host populations. Preliminary 3 color FACS experiments (V β subsets, CD4 or CD8, Thy 1.2) demonstrated a significant expansion of V β 11+ T cells, particularly CD4 cells, within the donor-derived population in the early weeks of GVHR. Modulation in V β 11 frequency and responsiveness in subsequent weeks is currently being investigated.

2) Role of CD8 cells in the immune response to *Toxoplasma gondii*

Toxoplasma gondii is an intracellular protozoan parasite. In man, chronic infections are normally asymptomatic; encysted parasites are controlled by the immune system. In immune-deficient individuals, such as infants, marrow transplant recipients, and AIDS patients, *Toxoplasma* can produce central nervous system damage and death. In collaboration with the

Laboratory of Parasitic Diseases, NIAID, we examined two models relevant for the mechanisms of immune resistance to *T. gondii*. In the first model, mice were vaccinated with non-virulent, temperature-sensitive mutant strain (TS-4) of *T. gondii*. Vaccinated mice survived subsequent challenge with a lethal dose of a virulent strain (RH). Spleen cells from vaccinated, but not those from untreated control mice were found to produce IL-2 and γ -interferon in response to irradiated *T. gondii* tachyzoites or to a preparation of soluble tachyzoite antigens. Both CD4 and CD8 cells produced these cytokines in vitro. Depletion of CD4 cells by in vivo treatment with antibody had no effect on resistance to *T. gondii*. Depletion of CD4 + CD8 or of CD8 T cells alone, however, abrogated resistance. Hence CD8 cells were crucial to host protective immunity in vaccinated mice.

Because CD8+ T cells are critical to 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 specifically kill syngeneic bone-marrow derived macrophages infected with TS-4 tachyzoites or pre-incubated with soluble *T. gondii* tachyzoites failed to generate significant CTL activity in vitro. Depletion of CD8+ cells from the effector cell population abrogated cytotoxic activity; depletion of CD4⁺ cells had little effect. CD8 depleted responder populations, even when supplemented with IL-2, lacked all CTL activity on infected targets. In contrast, CD4-depleted populations generated significant CTL activity, which was augmented by the addition of rIL-2 during the in vitro restimulation period. 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. Target killing is primarily restricted by genes mapping within the H-2D/L^d loci. These results establish MHC-restricted cytotoxicity as a major parameter of CD8 effector function against *T. gondii*.

In the second model, chronic *T. gondii* infection was established in B6D2F1 mice. Mice with chronic toxoplasmosis survive for up to 1 year. Host mice were made immune deficient by injection of parental C57BL/6 or DBA/2 lymphocytes into the F1 in order to generate a graft-vs-host reaction. Injection of C57BL/6 lymphocytes produces an acute suppressive GVHR, in which both CD4 and CD8 function is suppressed. In contrast, injection of DBA/2 lymphocytes produces a chronic B cell stimulatory GVHR in which only CD4 function is altered. Host mice undergoing an acute GVHR, induced by C57BL/6 cells, developed increased numbers of brain cysts, lost weight and died within 6 weeks. Mice undergoing a chronic GVHR, induced by DBA/2 cells did not show increased numbers of brain cysts and no mortality was observed within 12 weeks. Although both forms of GVHR produced deficits in *T. gondii*-specific responses, the deficit was more severe in the acute B6 induced GVHR.

This model of reactivation of chronic toxoplasmosis may be relevant to the disease process in AIDS patients. Acute toxoplasmosis appears only late in AIDS, corresponding to the period of loss of not only CD4, but also CD8 T cell function.

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:

Hakim FT, Sharrow SO, Payne S, Shearer GM. Repopulation of host lymphohematopoietic systems by donor cells during graft-versus-host reaction in unirradiated adult F1 mice injected with parental lymphocytes. J. Immunol. 1991;146:2108-2115.

Muluk SC, Hakim FT, Shearer GM. Enhancement of natural resistance to parental bone marrow grafts, by infection of F₁ host mice with murine cytomegalovirus. J. Immunol. 1990;145:1113-1119.

Muluk SC, Hakim FT, Shearer GM. Murine cytomegalovirus infection can enhance hybrid resistance through modulation of host natural killer activity. J. Immunol. 1990;145:1113-1119.

Via CS, Shanley JD, Shearer GM. Synergistic effect of murine cytomegalovirus on the induction of acute graft-vs-host disease involving major histocompatibility complex class I difference only. J. Immunol. 1990;145:3283-3289.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09263-10 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

mRNA Expression and Function of Cytotoxic T lymphocyte Granule Components

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

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S. Winslow Microbiologist EIB, NCI

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LAB/BRANCH

Experimental Immunology Branch

SECTION

Lymphocyte Cytotoxicity Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

2.0

OTHER:

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.)

We have studied the differentiation of resting CD8+CD4- thymocytes to become cytotoxic in short term in vitro culture after stimulation with immobilized antibodies against the T cell receptor. In the presence of IL4 and other lymphokines, strong proliferation and cytotoxicity was observed after 3 days.

We have analyzed the expression of genes for granule components associated with cytotoxicity as well as other genes expressed by stimulated CD8+ cells. The lymphokine-mediated increase in cytotoxic activity is accompanied by an increase in expression of the granule proteins cytolysin, granzymes A and B, and the serglycin proteoglycan core protein. In contrast, these conditions cause equal or lesser amounts of message for the lymphokines g-IFN, TNF, LT, and TY-5 compared to control cultures. If the activated CD8+ lymphocytes are re-exposed to surface-bound antibodies against the T cell receptor, they undergo a form of activation-induced cytotoxic response resulting in a metabolic decrease and death. During these studies we established a RT-PCR system to analyze these CD8+ cells for CD45 isoforms. Careful analysis of PCR products revealed significant amounts of a previously undescribed mRNA species in which exons 4,5,6, and 7 are all deleted. This message appears to be made to some degree by all lymphocytes analyzed and results in a very short region of extracellular ser/thr/pro-rich domain where most of the carbohydrate is attached. Lastly, human tumor-infiltrating lymphocytes (TIL) were also examined for expression granule protein mRNA using Northern blots. It was found that most TIL express mRNA for cytolysin, granzyme A, granzyme B and serglycin and are cytotoxic when tested in "redirected" assays in which MAB against the T cell receptor triggers the cytotoxicity. Occasional TIL are not cytotoxic and show poor expression of the cytolysin while expressing at least some other granule genes.

Project Description

Major Findings:

We have studied the differentiation of resting CD8+CD4- thymocytes to become cytotoxic in short term in vitro culture after stimulation with immobilized antibodies against the T cell receptor. Cytotoxic activity was measured with red blood cell target cells and aCD3xαDNP heteroconjugated antibodies to allow a polyclonal readout of a cytotoxic pathway assumed to require granule exocytosis and the granule cytolytic. Culture of these CD8+ T cells with immobilized anti CD3 gave rise to strong proliferation and cytotoxicity after 3 days. Cytotoxicity was not induced in the presence of anti-IL4, although cells became activated and proliferated vigorously. Cytotoxicity could be subsequently induced in these IL-4 deprived cultures within 2 days by addition of IL2, IL4 and "Con A supernatant" on day 4 of culture. Using PCR after reverse transcriptase (RT-PCR), we have analyzed the expression of genes for granule components associated with cytotoxicity as well as other genes expressed by stimulated CD8+ cells. The lymphokine-mediated increase in cytotoxic activity is accompanied by an increase in expression of the granule proteins cytolysin, granzymes A and B, and the serglycin proteoglycan core protein. In contrast, these CD8+ cells express equal or lesser amounts of message for the lymphokines g-IFN, TNF, LT, and TY-5 when cytotoxicity is induced than in control cultures. If the activated CD8+ lymphocytes are re-exposed to surface-bound antibodies against the T cell receptor, they undergo a form of activation-induced cytotoxic response resulting in a metabolic decrease and death. This response is prevented by cyclosporin A, suggesting a form of "programmed cell death". During these studies we established a RT-PCR system to analyze these CD8+ cells for the CD45 isoforms expressed during the transition from naive to activated to memory cells. Careful analysis of PCR products revealed significant amounts of a previously undescribed mRNA species in which exons 4,5,6, and 7 are all deleted. This message appears to be made to some degree by all lymphocytes analyzed and results in a very short region of extracellular ser/thr/pro-rich domain where most of the carbohydrate is attached. Lastly, human tumor-infiltrating lymphocytes (TIL) were also examined for expression granule protein mRNA using Northern blots. It was found that most TIL express mRNA for cytolysin, granzyme A, granzyme B and serglycin and are cytotoxic when tested in "redirected" assays in which MAb against the T cell receptor triggers the cytotoxicity. Occasional TIL are not cytotoxic and show poor expression of the cytolysin while expressing at least some other granule genes.

Proposed course:

We will study the differentiation of peripheral CD8+ T cells, both naive and memory as defined by CD44 expression, to become cytotoxic after cross-linking the TcR. We will test the lymphokine requirements and compare the activation of the granule and lymphokine genes with the naive CD8+ thymocytes described above. In addition we will compare the gene expression induced in CD4+ lymphocytes, which do not become cytotoxic after TcR crosslinking.

Analysis of CD45 expression in T lymphocytes by RT-PCR will be extended to human lymphocytes to confirm the existence of the form in which exons 3-7 are deleted.

A panel of mouse CD4+ Th1 and Th2 clones will be analyzed to see if this form is preferentially expressed in one versus the other subtype.

Publications:

Winslow SG, Henkart PA. Polyinosinic acid as a carrier in the microscale purification of total RNA. Nucl. Acids Res. in press, 1991.

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

PROJECT NUMBER

Z01 CB 09264-04 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Studies of T Lymphocyte Function in Transplantation

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

PI:	G.M. Shearer	Section Chief	EIB, NCI
Others:	M. Clerici	Visiting Associate	EIB, NCI
	B. Bermas	Medical Staff Fellow	EIB, NCI
	N. Seki	Special Volunteer	EIB, NCI
	A. Kosugi	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)

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 P. Kimmel, Renal Unit, George Washington University School of Medicine
 J. Mayes, Renal Department, University of Cleveland

LAB/BRANCH

Experimental Immunology Branch

SECTION

Cell Mediated Immunity Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.6

PROFESSIONAL:

1.8

OTHER

0.8

CHECK APPROPRIATE BOX(ES)

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

B

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

(Formally Titled: "Effects of Cyclosporin A on T Lymphocyte Regulation and Development")

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) and FK506, such that the CD4-sAPC is the most sensitive and CD4-aAPC is the least sensitive, when these drugs are added to cultures of PBL from healthy donors.

We tested the in vitro Th function of more than 150 human renal allograft recipients on multi-drug immunosuppressive therapy. Our results indicate that only the presence of a functionally 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.

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. First, we demonstrated that 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). Second, we 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. It appears that the in vitro Th test can detect the onset of a rejection episode up to two weeks earlier than assays of renal dysfunction such as an increase in creatinine. Using PBL from healthy donors, we have recently found that the new immunosuppressive drug, FK506, (which has been clinically more effective than CsA) exhibits a dose response curve that is similar to that of CSA for the CD4-aAPC and CD8-aAPC pathways. However, FK506 selectively abrogates the CD4-sAPC pathway at a 100-to-1000 lower concentration of drug than CsA. This finding demonstrates that FK506 more efficiently abrogates the relevant CD4-sAPC pathway than does CsA, but not the irrelevant CD4-aAPC and CD8-aAPC pathways. A multi-center protocol has been developed in which renal allograft patients will be given immunosuppressive drug adjustments, based on: a) standard renal functional assays in the control group; and b) our in vitro Th analysis in the experimental group. The objective of this study is to determine whether long-term renal function will be improved in the group of recipients whose drug adjustments are based on the immunologic assay rather than on renal functional tests. Studies are in progress to determine whether the rejection of other human solid organ allografts such as heart liver and pancreas follow a similar pattern.

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 in press, Aug. 1, 1991.

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

PROJECT NUMBER

Z01 CB 09265-10 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Investigator	EIB, NCI
	M. Vacchio	Bio. Lab. Tech.	EIB, NCI
	S. Sharrow	Senior Investigator	EIB, NCI
	R. Karpati	Biotechnology Fellow	EIB, NCI
	S. Rosenberg	Chief	SB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

(Formally Titled: "Analysis of the T Cell Alloreactive Repertoire")

The T cell receptor (TCR) repertoire expressed in mice of different genetic origins was analyzed by flow cytometry and by analysis of TCR-specific mRNA. When TCR V β usage was assessed, strain-specific decreases were detected in expression of 12 of the 22 known mouse V β products. In each case studied, V β deletion was dominant in F1 mice bred between high and low expressors, indicating that these deletions represent negative selection of potentially self-reactive T cells in mice expressing the corresponding self ligands. These V β deletions fail to occur in athymic nude mice, demonstrating that the thymus is critical in mediating self tolerance by negative selection.

The self ligands mediating V β -specific negative selection were analyzed. In most instances, both major histocompatibility complex (MHC)-encoded and non-MHC-encoded components of the deleting ligand were identified. In some instances, the deleting ligand corresponded to a known endogenous superantigen, for example in the demonstration that Mls^C is the ligand for V β 3-expressing T cells. In other instances, previously unknown superantigens were identified on the basis of observed V β deletions, e.g. the demonstration of Mls^F as the ligand for V β 11- and V β 12-expressing T cells. In several cases, a novel "genetic redundancy" was identified in the non-MHC ligands for V β deletion, such that any one of two or more unlinked genes was permissive for deletion.

Analysis of TCR V β expression has been used to characterize the in vivo response of mice to syngeneic tumors. A non-random but nevertheless highly heterogeneous T cell response was observed in tumor-infiltrating lymphocytes.

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. The Mls system of endogenous superantigens is thus composed of the products of multiple non-allelic and unlinked genes, with no evidence of polymorphism at any locus. An influence of MHC polymorphism upon the stimulatory activity of Mls^a, Mls^c, and Mls^f was demonstrated.

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. This process of negative selection was studied by examining expression of each of the 22 known TCR V β gene products in a large panel of inbred mouse strains. V β expression was studied by measuring mRNA specific for each V β family, and when possible by flow cytometry employing V β -specific mAb. It was found that significant strain-specific decreases in expression occur in at least 12 of the 22 V β products and that each of these deletions is dominant in F₁ mice, consistent with the conclusion that these deletions occur in the process of eliminating T cells with potential reactivity for self determinants. The role of the thymus in mediating TCR negative selection was analyzed by studying congenitally athymic nude mice. A comparison of T cell receptor V β expression in congenic pairs of normal and athymic mice indicated that the normal V β 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 V β -specific negative selection.

When the self ligands responsible for these deletions were analyzed, it was demonstrated that MHC and non-MHC products appear to be involved in each deletion. In some instances, non-MHC ligands corresponded to known endogenous superantigens, e.g. in the demonstration that Mls^c is recognized by T cells expressing V β 3 and that Mls^c expressed as a self antigen is a deleting ligand for V β 3-expressing T cells. In other instances, new superantigens were discovered on the basis of V β -specific deletions, e.g. in the identification of Mls^f as a ligand for V β 11 and V β 12 deletion segments. Selection of the T cell repertoire may therefore be detected by analysis, not of V β expression alone, but by expression of particular α chain/ β chain pairs. The expression of specific Va/V β pairs by T cells was analyzed by flow cytometry. It was found that Va's and V β 's are not randomly associated on peripheral T cells. Moreover, patterns of Va/V β pairing appear to differ between inbred mouse strains, suggesting that TCR repertoire selection influences this expression.

4) TCR expression in tumor-infiltrating lymphocytes (TIL).

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 $\alpha\beta$ expression was observed in the vast majority of TIL. $V\beta$ usage was heterogeneous in these populations. The pattern of $V\beta$ usage in TIL differed significantly from that observed in splenic T cells from the same strain but no association was found between $V\beta$ usage and the specificity of TIL for a particular syngeneic tumor.

Proposed Course of Research:1) Analysis of ligands mediating $V\beta$ -specific negative selection.

Recent publications from several other laboratories have demonstrated that endogenous or exogenous mouse mammary tumor virus (MTV) products may be the ligands for $V\beta$ -specific selection. Similar results have been generated in this laboratory. Additional aspects of this study are in progress:

a) Identification of MTV product. Mapping and transfection studies have identified a role of the MTV LTR gene in $V\beta$ deletion, but have not demonstrated whether or not the product of this gene is directly involved in T cell recognition or deletion. In collaboration with Dr. 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 $V\beta$ -specific T cell activation in vitro and clonal deletion or inactivation in vivo.

b) Mapping of additional $V\beta$ ligands. To date, all of the characterized endogenous mouse $V\beta$ deleting ligands have been mapped to endogenous MTV genes. Recent studies in this laboratory have identified involvement of non-MHC genes in the previously uncharacterized deletions of $V\beta 16$ and $V\beta 19a$. The relevant non-MHC genes will be mapped by segregation analysis, including a study of linkage to endogenous mouse retroviral sequences. In collaboration with Dr. Robert Callahan (NIH), TCR $V\beta$ expression has been analyzed in an inbred strain of feral mice that has no identifiable endogenous or exogenous MTV genes. Preliminary experiments suggest that a $V\beta$ -specific deletion occurs in this strain. More extensive analysis will be carried out to characterize this deletion, to determine whether it is mediated by an endogenous or exogenous gene product, and to identify the relevant gene.

Publications:

Vacchio MS, Ryan JJ, Hodes RJ. Characterization of the ligand(s) responsible for negative selection of $V\beta 11$ - and $V\beta 12$ -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.

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.

Hugin AW, Vacchio MS, Morse HC. A virus-encoded "superantigen" in a retrovirus-induced immunodeficiency syndrome of mice. *Science* 1991;252:424-427.

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" V β s, V β 5, v β 11, and V β 12 T Cell Receptors for Antigen. *J. Immunol.* 147: In press, 1991.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09266-09 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

S. Murakami Visiting Fellow EIB, NCI

Q. Vos Visiting Fellow EIB, NCI

COOPERATING UNITS (if any)

Naval Medical Research Institute
Food and Drug Administration
Oklahoma Medical Research Institute

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The supernatants of activated type 2 T helper cell (Th2) clones are capable of inducing polyclonal proliferation and immunoglobulin (Ig) secretion by heterogeneous unprimed B cell populations. Studies employing recombinant lymphokines demonstrated that IL5 is sufficient to induce these responses. Recombinant IL5 stimulation also results in the appearance of a phenotypically novel B cell population which expresses high densities of Pgp-1 (CD44) and relatively low densities of B220 (CD45) and Ia. Cell fractionation experiments demonstrated that the B cell subpopulation expressing this novel phenotype mediates nearly all of the proliferative and immunoglobulin secretory activity of the activated B cell populations. In addition, the CD44 expressed by these cells is capable of mediating binding to the extracellular matrix material hyaluronic acid (HA), indicating a potential role for CD44 in regulating the trafficking of activated B cells in vivo. The CD44 molecules expressed on IL5-stimulated B cells migrate with a lower molecular weight than does CD44 expressed by control B cells, reflecting differential glycosylation.

A series of mAb was generated by immunizing rats with activated mouse B cells. One of these mAb (GL7) reacts by flow cytometry with a subpopulation of B cells activated with stimuli including LPS or anti-Ig. This mAb precipitates a 29-31 KDa molecule from activated but not resting B cells. This appears to represent a previously undescribed activation molecule.

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.

Project Description

Major Findings:

1. CD44 expression and B cell activation.

Culture of heterogenous unprimed B cells with the supernatant of activated Th2 clones resulted in B cell proliferation, polyclonal Ig secretion, and phenotypic changes in the B cell population. Exposure to recombinant IL5 was sufficient to induce both proliferation and Ig secretion. In addition, IL5 stimulation resulted in the appearance of a B cell population which is surface Ig bright, Pgp-1 (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.

³⁵S-methionine metabolic labeling and ¹²⁵I 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.

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 (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.

2. 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. During characterization of the transgenic mice used in these studies, it was noted that an unusually high proportion of serum Ig molecules of endogenous (non- μ/k) origin expressed the transgene idiotype, suggesting either mixed isotype Ig molecules, extensive class switching by trans-rearrangement, or a "network" influence on Ig expression.

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. Mechanism of Th cell-B cell interaction.

The antigen-specific interaction of Th cells and B cells will be analyzed by a digital imaging system in which intracellular $[Ca^{++}]$ can be analyzed over time in individual Th/B cell conjugates. In this system, the ability of cell interactions to signal each of these populations will be analyzed. Subsequently, the role of antigen-specific and non-specific cell interaction molecules will be analyzed by testing the effects of mAb specific for such molecules.

The regulation of idiotype expression in Ig transgenic mice will be further analyzed. It will be determined whether the transgene idiotype detected on endogenous (non- μ/k) Ig molecules results from a class switching or splicing event that involves rearrangement of the transgenic V region from the transgenic μ to an endogenous C region, or whether the idiotype is expressed on a completely endogenous Ig molecule. The latter possibility would be consistent with the controversial existence of a network influence on Ig idiotype selection. Initial approaches will include the use of ELISA techniques and radiation bone marrow chimeras to determine whether transgene idiotype is expressed only on cells expressing the transgene. Definitive analysis will be based upon PCR amplification and sequencing of Ig cDNA.

Publications:

Guy R, Ullrich SJ, Foo-Phillips M, Hathcock KS, Appella E, 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; in press.

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, Kincade PW, Hodes RJ. The functional role of CD44 (Pgp-1) on activated B cells. Immunol. Res.:in press.

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. 146:1422-1427.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09267-09 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	M. Clerici	Visiting Associate	EIB, NCI
Others:	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)

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 R. N. Boswell, 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:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B,A,D

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

(Formally Titled: "T Cell Immune Function in Asymptomatic HIV Seropositive Patients")

Peripheral blood leukocytes (PBL) from AIDS patients but not from asymptomatic HIV-infected (HIV+) individuals exhibit two distinct types of antigen presenting cell (APC) defects. PBL from children infected with HIV exhibit the same complex spectrum of T helper cell (Th) defects that we reported in adult patients. PBL from healthy HIV- children do not generate Th responses to recall until 2 years of age, although T cell responses to HLA alloantigens (ALLO) and phytohemagglutinin (PHA) are strong at birth. The early defect in Th responses to recall antigens seen in HIV+ adults and children is associated with a non-HIV inhibitory factor that is produced by CD8+ cells and is selective in its action in that it only suppresses HLA self-restricted Th responses. PBL from volunteers immunized with a candidate AIDS vaccine generate potent HIV-specific T helper and effector responses, even one year after immunization. Using our in vitro Th tests, we can detect long-term improvement in Th function in more than 50% of patients on AZT, ddI, or soluble CD4-IgG therapy. We can detect evidence of HIV exposure by sensitive HIV-specific T cell tests, using PBL from high risk HIV seronegative cohorts who are also HIV- by the polymerase chain reaction.

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, β 2-microglobulin and antigenic peptide.

Project Description

Major Findings

Our previous study of asymptomatic HIV-infected (HIV+) individuals indicated a complex spectrum of Th defects but not a defect in APC function. We have subsequently investigated APC function in patients with AIDS, and have found two types of APC defects in approximately 2/3 of AIDS patients: 1) a defect in antigen presentation but not in ability to stimulate a mixed lymphocyte reaction (MLR); and 2) a defect in MLR stimulating ability that is associated with a 10-fold reduction in HLA class II antigen expression on macrophages (but not on other cell types). These results indicate that the immune deficiency of AIDS patients is not limited to Th defects, but can also include defects in APC function.

We have found that PBL from the majority of HIV+ patients contain cytotoxic T lymphocytes (CTL) that are HIV-specific and HLA-self restricted. Unlike CTL in other murine and human systems, HIV-specific cytolytic activity can be detected directly from blood without in vitro restimulation.

We have demonstrated that the selective defect reported in Th function to recall antigens is due to an inhibitory factor that is produced by CD8+ T lymphocytes on short-term (24 hour) culture. Although this factor has not been identified or characterized, it appears not to be HIV nor a product of the virus. The factor is produced in both asymptomatic and AIDS patients, even patients on anti-retroviral drugs.

PBL from HIV-infected children with and without AIDS symptoms were tested in vitro for defects in Th function. The same spectrum of defects that we reported in HIV+ adults was also observed in children. In order to determine whether the absence of Th function was the result of HIV infection or was due to the lack of immunologic maturation and/or absence of previous antigenic exposure, PBL from children of different ages were tested for Th and APC function. We found that Th function to ALLO and PHA is developed at birth, but that Th function to recall antigens such as tetanus does not develop until 24 months of age. Studies are in progress to establish whether the defect in the Th responses of infants is due to immaturity of Th and/or APC, and to determine the nature of the defect(s). Identification of the functional deficiencies outlined above in pediatric AIDS and healthy infants will provide information that is relevant for assessing the immune responses of infected children on anti-retroviral therapy (see below), as well as for evaluating immune function in children born of HIV-infected mothers. We are currently evaluating the Th responses of HIV+ pregnant women in an attempt to identify immunologic parameters that will be predictive for mother-to-fetal HIV transmission and to assess the immune status of such HIV-infected infants of the first six pregnant females test five were responsive to HIV synthetic peptides, of the first three neonates tested one was responsive to these same peptides.

The Th function of PBL from patients on anti-retroviral drug protocols of AZT, ddI, and soluble CD4-IgG was assessed before, and during therapy. We observed

improvement in both short-term (within 8 weeks) and in long-lasting (up to 120 weeks) in Th function: a) in 75% of the patients either on AZT or on soluble CD4-IgG (both adult studies); and b) in 60% of patients (pediatric) on ddI therapy. These changes of in vitro Th function were observed without there being a concomitant increase in CD4 cell numbers.

The Th functional parameters that we developed to monitor progression toward AIDS (an earlier study), and now used to monitor drug efficacy is being tested as a possible surrogate marker by the AIDS Clinical Trials Group of the NIAID.

Using PBL from uninfected volunteers multiply immunized with low doses (40-80 μ g) of an HIV recombinant gp160 (rgp160) candidate AIDS vaccine, we have demonstrated strong T helper and cytotoxic (CTL) responses to synthetic peptides of HIV envelope. Such responses were long-lasting in that they could be detected for more than one year after the last boost, and reinmunization further enhanced T cell immunity. It is noteworthy that, at these low immunization doses, antibody responses to HIV were absent or transient. These results suggest that it may be possible to develop a safe and effective AIDS vaccine.

Based on our experience (above) of low dose immunization, with HIV antigens, we investigated the possibility that a proportion of those individuals who are at high risk for HIV infection but who have remained HIV-seronegative will exhibit evidence of exposure to HIV antigens by our HIV-specific in vitro T cell assays. Among two groups of individuals who fit the above criteria (i.e., homosexual men and i.v. drug abusers who have remained seronegative during the past decade despite continued high risk behavior), we have observed a high proportion whose PBL generate strong HIV peptide-specific Th and CTL responses. Approximately 1/3 of the homosexual group seroconverted during a 12-month follow-up. We have tested the PBL from more than 100 individuals considered not to be at risk for HIV infection. Only one individual was positive by our T cell tests. This individual was a laboratory worker who received a needle stick three years earlier and has remained seronegative. Preliminary results of a study of health careworkers who have had parenteral exposure to HIV but have test seronegative indicate a significant proportion who are HIV-specific T cell reactive. It is noteworthy that the PBL of the homosexual men tested in this study also negative for HIV by the polymerase chain reaction (PCR). However, those individuals who seroconverted also became PCR+ at the time of seroconversion. These results indicate that we can detect HIV-specific T cell activity in PBL of exposed individuals up to one year before evidence of infection is observed by antibody or PCR. Our findings raise the possibility that we are observing a period of immune-protection, in which T cell immunity (but not antibody) is keeping the virus under control.

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 β 2-

microglobulin at 37°C. These results suggest a defect in the stability of the immunogenic complex formed by self class I; β 2-microglobulin and peptide in WAS patients, and provide a useful human model for investigating antigen presentation.

Publications:

Fuchs D, Shearer GM, Boswell RN, Clerici M, Reibnegger G, Zajac RA, Wachter H. Activated cell-mediated immunity and reduced proliferative response of T cells in vitro in patients with HIV-1 infection. Clin. Immunol. Immunopath. 1990;80:44-48.

Ahlers J, Clerici M, Hosmalin A, Shearer GM, Berzofsky JA. T Helper Cell Responses In Techniques in HIV Research, A. Aldovini and BD. Walker, (Eds.): Stockton Press, New York, pp 211-222;1990.

Clerici M, Landay A, Kessler HA, Zajac RA, Boswell RN, Muluk SC, Shearer GM. Multiple patterns of alloantigen presenting/stimulating cell dysfunction in patients with AIDS. J. Immunol. 1991;146:2207-2213;1991.

Clerici M, Lucey DR, Zajac RA, Boswell RN, Gebel HM, Takahashi H, Berzofsky JA, Shearer GM. Detection of cytotoxic T lymphocytes specific for synthetic peptides of gp160 in HIV-seropositive individuals. J. Immunol. 1991;146:2214-2219.

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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. in press, 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 in press, 1991.

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 in press, 1991.

Clerici M, Shearer GM. Cellular Immunology of HIV infection. Clinical Immunology Newsletter, in press, 1991.

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. in press, 1991.

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

PROJECT NUMBER

Z01-CB-09268-04 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Role of CD4 and CD8 Accessory Molecules in T Cell Function

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

PI: A. Singer Chief EIB, NCI

Others: T. Nakayama Visiting Fellow EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL

OTHER

1.25

1.25

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

B

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

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 and assembled TCR complexes, and that CD4 mediated signals regulate the TCR degradation rate in CD4⁺CD8⁺ thymocytes.

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 mobilization. We found that surface TCR complexes on "uninduced" CD4⁺CD8⁺ thymocytes that had not yet escaped from CD4-mediated inhibition signaled very poorly as measured by calcium mobilization, whereas TCR on "induced" CD4⁺CD8⁺ thymocytes that had been released from CD4-mediated inhibition mobilized calcium as well as mature T cells. The relative inability of TCR on uninduced CD4⁺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:

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.

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⁺CD8⁺ thymocytes by CD4. Science 1990;249:1558-1561.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09270-08 E

PERIOD COVERED
October 1, 1990 to September 30, 1991

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)
Regulation of Expression of MHC Class I Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
P.I.: Dinah Singer Section Chief EIB, NCI

Others:

Jocelyn Weissman	Chemist	EIB, NCI
Kevin Howcroft	IRTA	EIB, NCI
Lisa Palmer	IRTA	EIB, NCI
Jeff Richardson	IRTA	EIB, NCI

COOPERATING UNITS (if any)
W.I. Frels, Agricultural Research Service, USDA

LAB/BRANCH
Experimental Immunology Branch

SECTION
Molecular Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 4	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.)

MHC class I genes encoding transplantation antigens are ubiquitously expressed, although their level of expression varies among tissues. Analysis of the 5' flanking DNA sequences 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 elements. One of the regulatory elements (RE-140) associated with this gene consists of overlapping negative and positive elements. Introduction into transgenic mice of a series of nested deletions containing a common 3' terminus, but differing in the extent of 5' flanking regulatory sequences, reveals that this complex regulatory element functions in a tissue specific manner, such that the enhancer activity predominates in lymphoid tissues, but not in non-lymphoid tissues. Another negative regulatory element, RE-105, has been identified which is distinct from RE-140 in both sequence and function. In transgenic animals, RE-105 functions as a silencer in all tissues examined.

The silencer component of RE-140 has been shown to have striking similarities to the yeast mating type repression system. A moderate DNA sequence homology exists between the yeast and mammalian operators. Mammalian factors bind to the yeast $\alpha 2$ operator and also specifically interact with a yeast $\alpha 2$ -binding protein. Further, the yeast $\alpha 2$ operator functions as a silencer element in mammalian cells when placed upstream of a MHC class I promoter.

All of the functionally identified regulatory elements are associated with trans acting factors. RE-140 DNA forms distinct enhancer and silencer associated complexes with cellular factors. Each consists of at least two distinct factors. Analysis of binding activity from a variety of cell lines and tissues reveals that RE-140 enhancer binding activity is present in all extracts, independent of the level of class I expression. The level of RE-140 silencer binding activity is inversely proportion to the level of class I gene expression. These studies have led to the proposal that class I genes are negatively regulated.

Project Description

Major Findings

Expression of individual MHC class I genes is actively regulated: large differences in the levels of class I gene expression are observed among tissues. Thus, expression is high in lymphoid tissues, but low in other tissues such as kidney and liver. However, even among the lymphoid tissues, there are distinct differences in the level of expression, such that B cells express twice as much class I as do T cells. In earlier studies, we demonstrated that introduction of one of the swine class I genes, PDI, into a transgenic mouse resulted in its regulated expression, in a pattern indistinguishable from that observed *in situ* in the pig. These studies indicated that regulatory sequences responsible for establishing normal patterns of expression were contained within the transgene. To further define the regulation of this class I gene, we have undertaken a detailed analysis of the 1.1 kb of 5' DNA sequences flanking the PDI promoter, and have identified a series of positive and negative regulatory elements.

Using a series of 5' deletion mutants, as well as discrete DNA segments, ligated to the reporter gene CAT, we have identified the transcriptional promoter, the interferon response element, and an array of positive and negative regulatory elements. Of particular interest is the existence of two regulatory elements. One, RE-140, maps between -700 and -800 bp, while the other, RE-105, is located between -400 bp and -500 bp. RE-105 is a negative regulatory element, whose removal results in increased transcription from the down-stream promoter of about 2-fold, in transfected cell lines. Its *in vivo* function similarly appears to be as a silencer element, since in all tissues of transgenic mice there is greater transgene expression in the absence of RE-105 than in its presence.

RE-140 is a complex regulatory element consisting of two overlapping functional elements: a silencer and an enhancer. The enhancer is comprised of an interrupted, inverted repeat, whereas the silencer consists of two discontinuous segments. The enhancer element is not homologous to any other known regulatory element. However, the silencer element displays weak homology with the yeast $\alpha 2$ mating type operator. Indeed, these two negative regulatory systems have been shown to have striking similarities. Thus, the same mammalian factors that bind to the RE-140 silencer also bind to the yeast $\alpha 2$ operator, and conversely the RE-140 silencer element can compete the binding of yeast factors to the yeast $\alpha 2$ operator. Further, the yeast $\alpha 2$ operator functions as a silencer element in mammalian cells when placed upstream of a MHC class I promoter.

RE-140 displays cell-type and tissue specificity. In cells which express low levels of class I, such as kidney cells, RE-140 functions as a silencer element. However, in cells which express high levels of class I, such as lymphoblastoid cells, the enhancer activity predominates. Tissue specific

differences are clearly evident in transgenic mice, where it is observed that the element functions as an enhancer in lymphoid tissues, but not in non-lymphoid tissues.

The differences in activity of RE-140 correlate with the levels of silencer binding factors in different cell types and tissues. Discrete complexes are generated with the silencer and enhancer elements. Enhancer binding factors are constitutively expressed in all cell types. However, differences in the level of silencer binding factors are observed that are inversely correlated with the level of class I expression. Thus, low class I expressing cells contain greater amounts of silencer binding activity than high class I expressers, suggesting that this silencer factor plays a role in establishing tissue specific differences in class I transcription. The silencer binding factor is labile, allowing for rapid activation of class I genes in response to external stimuli.

Initial studies aimed at characterizing the trans acting factors reveal that each component of RE-140 binds a complex of at least two proteins. None of the proteins which bind RE-140 can bind independently, but must bind as a complex. Further studies are in progress to define these proteins.

RE-105, which functions as a silencer in all tissues, forms complexes with extracts from all cell types tested. The relative abundance of these complexes does not appear to differ as a function of the intrinsic level of expression. As in the case of RE-140, a complex of factors associates with RE-105; separated factors are incapable of binding the element. Surprisingly, at least some of the factors which bind RE-105 are common to those which bind RE-140. Since RE-105 functions as a silencer in all cell types examined, it does not appear to play a role in establishing tissue specific patterns of class I expression. However, it may function to regulate cellular responses to exogenous stimuli, which cause changes in intracellular levels of known transcriptional factors. Thus, we have recently demonstrated that elevation of serum in cell cultures reduces class I expression. This suppression has been mapped to RE-105.

Studies are in progress to further characterize the trans acting factors which associate with RE-105 and RE-140, and the relationships among these factors.

Publications:

Singer DS, Maguire JE. Regulation of expression of Class I MHC genes. CRC Reviews in Immunology, Volume 10, 1990.

Maguire JE, Ehrlich R, Frels WI, Singer D. Regulation of expression of a Class I major histocompatibility complex transgene. J. Reprod.Fert. Suppl. 1990;41:59-62.

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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, in press.

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Accolla RS, Auffray C, Singer DS, Guardiola J. The Molecular Biology of MHC Genes. Immunology Today 1991;12:97-99.

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

PROJECT NUMBER

Z01 CB 09273-04 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

T Cell Differentiation and Repertoire Selection

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

PI:	A. Singer	Chief	EIB, NCI
Others:	E.W. Shores	IRTA	EIB, NCI
	J. Roberts	Guest Researcher	EIB, NCI
	Y. Takahama	Visiting Fellow	EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

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

B

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

We 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 identifiable 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 β 8 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.

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 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 Thyl⁺ thymocytes from most scid mice contain only CD4⁻CD8⁻ (double negative) TcR⁻ cells. These cells are IL-2R⁺ and Lyl dull, and so are similar to double negative cells from the thymic of normal mice. However, upon the introduction of TCR⁺ cells into the thymic 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-6C⁺. Interestingly, both subsets appear late in ontogeny and have identically skewed TCR repertoires characterized by over-expression of V β 8 and expression of autoreactive TCR. However, the two subsets are clearly not identical, as Ly-6C⁺ thymocytes represent a readily identifiable subpopulation within each CD4/CD8 thymocyte subset, including the mature CD4⁺ single positive thymocyte subset. Nevertheless, the Ly-6C⁺ thymocytes within the CD4⁺ thymocyte subset express a skewed TCR repertoire that is identical to that of other Ly-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-6C⁺ thymocytes appear to represent the immediate precursors of CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ thymocytes, but appear to be unrelated to the Ly-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:

Takahama Y, Kosugi A, Singer A. Phenotype, ontogeny, and repertoire of CD4⁻CD8⁻ T cell receptor ($\alpha\beta^+$) thymocytes: Variable influence of self-antigens on TCR-V β usage. J. Immunol. 1991;146:1134-1141.

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.

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. In press.

Takahama Y, Sharrow SO, Singer A. Expression of an unusual T cell receptor (TCR) V β 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. Submitted.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09275-04 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

In Vivo Study of MHC-Specific T Cells

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

PI: A. Singer Chief EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

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TOTAL MAN-YEARS:

1.25

PROFESSIONAL:

1.25

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.)

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 allografts across a class II MHC barrier requires the production of endogenous IFN γ , 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.

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 T_k cells that assess individual cells in the dermis of the graft for expression of foreign histocompatibility antigens. The in vivo T_k 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 T_k 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 allograft rejection in these cases was the generation of a novel population of CD8⁺ T_k 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:

Rosenberg AS, Finbloom DS, Maniero TG, Van der Meide PH, Singer A. Specific prolongation of MHC class II disparate skin allografts by in vivo administration of anti-IFN γ monoclonal antibody. *J. Immunol.* 1990;144:4648-4650.

Rees MA, Rosenberg AS, Singer A. Characterization of T cell subsets mediating rejection of established fetal pancreas grafts and failure to observe graft adaptation. *Transplantation* 1990;49:1130-1133.

Rosenberg AS, Rees MA, Munitz TI, Singer A. In vivo model of antigen specific transplantation tolerance. In Lotze, M.T. and Finn, O.J. (Eds.): *Cellular Immunity and the Immunotherapy of Cancer*. Wiley-Liss, New York, pp 193-205, 1990.

Rosenberg AS, Munitz TI, Maniero TG, Singer A. Cellular basis of skin allograft rejection across a class I major histocompatibility barrier in mice depleted of CD8⁺ T cells in vivo. *J. Exp. Med.*

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

PROJECT NUMBER

Z01 CB 09279-05 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Isolation and Characterization of non-classical H-2 class I genes

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

P.I.: Dinah Singer Senior Investigator EIB, NCI
 Others: Kevin Howcroft IRTA EIB, NCI
 Stuart Rudikoff Senior Investigator LG, NCI

COOPERATING UNITS (if any)

William Frels, Agricultural Research Service, USDA

LAB/BRANCH

Experimental Immunology Branch

SECTION

Molecular Regulation Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.6

PROFESSIONAL

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

(Formally Titled: "Isolation and Characterization of a Novel H-2 Class I Gene")

A highly divergent member of the MHC class I gene family, M1, is representative of a small sub-family of three sequences. M1 itself appears to be a direct descendant of a primordial class I gene, which antedates speciation. M1 has been mapped to a region of chromosome 17 telomeric to Qa and has been used to define a new subregion M. The other two members of the sub-family, M2 and M3, are also highly divergent from the rest of the class I family, but are also divergent from M1 and one another. Like M1, both M2 and M3 contain open reading frames and legitimate splice sites in all exons. M1 appears to contain a functional promoter, since introduction into fibroblasts of a 3.4 kb fragment of DNA containing the M1 coding sequences results in M1 expression. Similarly, the M1 promoter, ligated to the CAT reporter gene, is able to direct CAT enzyme expression. However, M1 is not detectably transcribed *in vivo* in a variety of cell lines or adult tissues, as assessed by PCR analysis. Expression of M1 is controlled by a strong silencer element located within a 16 kb genomic fragment containing the M1 gene. The patterns of expression of M2 and M3 are under investigation. No M1 antigen has been identified to date. To attempt to generate M1 product, transgenic mice have been produced in which M1 expression is directed by a viral LTR promoter. Similar transgenic lines have been produced using the truncated M1 promoter. Analysis of the expression of M1 in these lines is in progress.

Project Description

Major Findings:

The murine MHC class I gene family consists of the classical class I genes, encoding transplantation antigens, as well as a large number of non-classical class I genes, encoding products whose functions are not completely defined. Two sub-regions of the MHC encoding such non-classical genes were originally defined by serology: Qa and Tl. Recently, a third subregion, M, has been defined by isolation of a non-classical class I gene, M1. M1 is the first gene to define this region; others which map to the same region are HMT and thy 19.4.

Although by DNA sequence analysis M1 appears to have the capacity to encode a product, it has not been possible to identify such a product. M1 transcripts are not detected by PCR amplification in either lymphoid or non-lymphoid cell lines or in adult somatic tissues. The regulation of M1 expression is currently under investigation. Introduction into mouse fibroblasts of the M1 gene within a 16 kb genomic fragment of DNA does not result in the expression of the gene. However, transfection with a 3.4 kb derivative, containing all of the M1 coding sequences, but a truncated 5' flanking sequence, does generate M1 transcripts. These observations suggest the presence of a negative regulatory element; the precise location and sequence of this element are being determined. Transgenic mice containing the 3.4 kb genomic fragment of M1 have been generated and are being characterized. To generate the M1 product, M1 coding sequences have been placed under the control of a viral LTR promoter; this construct has also been introduced into transgenic mice to obtain expression of M1.

M1 is a member of a small sub-family of three DNA sequences. The remaining two members, M2 and M3, have been isolated and their DNA sequences determined. M1 is more related to M2 than to M3; in the most conserved exon, exon 4, M1 and M2 diverge by only 7.4% whereas M1 and M3 diverge by 20.4%. The genomic locations of M2 and M3 are being determined. Like M1, M2 and M3 sequences contain completely open reading frames in all of the exons and legitimate splice sites. Analysis of the predicted protein sequences of the three genes reveals that critical residues important in maintaining class I structure have been conserved in all three sequences. Studies are in progress to examine whether M2 or M3 are expressed in vivo.

Publications: None

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09281-06 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any)

Naval Medical Research Institute
Food and Drug Administration

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF OBJECTIVES AND APPROACH (Type appropriate to the project and type of cells involved)

Subpopulations of T helper and thymic T cells were defined by flow cytometric analysis, including an analysis of CD45 isoform expression using newly developed mAb that detect CD45 exon-specific epitopes. The responses of these T cell subpopulations to T cell receptor (TCR) cross-linking were characterized by measurement of intracellular $[Ca^{++}]$. Co-cross-linking of CD45 and the TCR resulted in down-regulation of the $[Ca^{++}]$ response of mature T cells to TCR signalling, and a less pronounced effect on responses of thymocytes.

The effect of prior activation history on subsequent responses of cloned T cells to TCR-mediated stimuli was examined. The T helper type 1 (Th1) line AE7.6 was maintained in vitro by repeated stimulation with IL2 alone or by stimulation with specific antigen and APC in addition to IL2. Cell lines carried under both conditions proliferated equivalently in response to stimulation with anti-CD3 antibody. However, this stimulation induced strong phosphatidyl inositol (PI) hydrolysis and increased intracellular $[Ca^{++}]$ only in cells that had been maintained by stimulation with IL2 alone; cells that had been repeatedly stimulated with specific antigen + APC generated neither PI nor Ca^{++} responses. The signaling pathways utilized by cloned T cells were therefore influenced by prior stimulation through the TCR.

Regulation of IL3, IL4, IL5, and GM-CSF gene expression by type 2 helper (Th2) clones was studied in responses to multiple inducing stimuli and pharmacologic inhibitors. It was found that independent regulation of discrete lymphokine genes can occur and is dependent upon the inducing stimulus. In particular, expression of IL4 and IL5 genes, which are generally co-expressed by Th2 clones in response to TCR-mediated stimuli, can be independently regulated.

Project Description

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 intracellular $[Ca^{++}]$ 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) Regulation of lymphokine expression.

It was found that the pattern of lymphokine genes expressed by Th2 clones was

dependent upon the stimulus used to activate the T cells. Some lymphokines, such as IL5, were induced in response to anti-CD3 as well as in response to recombinant IL2 stimulation, whereas IL4 gene expression by the same clone was induced by anti-CD3 but not recombinant IL2. The regulation of IL4 and IL5 gene expression in response to T cell receptor mediated stimulation was analyzed using pharmacologic agents. It was found that cyclosporine A completely inhibited the induction of IL4 mRNA by anti-CD3 stimulation, but did not have the same effect on IL5 gene expression. Reciprocally, the protein synthesis inhibitors cycloheximide and anisomycin completely inhibited induction of IL5 but not IL4 gene expression. These findings indicated that IL4 and IL5, which are generally expressed in parallel by Th2 cells, can be independently regulated.

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.

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.

Publications:

Bohjanen P, Okajima M, Hodes RJ. Differential regulation of interleukin 4 and interleukin 5 gene expression. A comparison of T cell gene induction by anti-CD3 antibody or by exogenous lymphokines. Proc. Natl. Acad. Sci. USA 1990;87:5283-5287.

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

PROJECT NUMBER

Zol CB 09282-05 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	G. C. Tsokos	Senior Investigator	ARB, NIADDKD
	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:

2.4

1.7

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.)

(Formally Titled: " T Cell Immune Deficiency in Mice and Humans with Autoimmune Cells")

Thyroid epithelial cell-specific CD4⁺ and CD8⁺ T cell lines were isolated from thyroid infiltrates of mice undergoing experimental-induced autoimmune thyroiditis. These cell lines proliferate and generate IL-2 in response to thyroid antigens and exhibit 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 V β usage.

Patients with systemic lupus erythematosus (SLE) can exhibit both T helper cell (Th) and antigen presenting cell (APC) defects. The Th defects are complex in that different patients have been identified whose peripheral blood leukocytes (PBL) are: 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.

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-2K^k and H-2D^b. TCR- $\alpha\beta$ analyses indicated a broad spectrum of V β usage, with V β ⁶, V β 8.1, and V β 13 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 erythematosus (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.

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. Although we have not followed the group of patients long enough to correlate a particular pattern of in vitro Th function with disease status (for example SLE flares), we have observed flaring with restoration of complete Th function in a few patients followed longitudinally. Thus, it is possible that we shall be able to predict the onset of an SLE flare by these in vitro Th tests. Studies are in progress to determine the mechanism(s) responsible for this series of Th defects. We have also found that approximately 80% of SLE patients exhibit a defection APC function similar to that we observed in AIDS patients.

Because of the parallels between the spectra of Th defects seen in SLE patients and asymptomatic HIV-infected individuals, and the similarities in the APC defect observed in SLE patients and in patients with AIDS, approximately 60% of this project is considered to be AIDS-related research.

Publications:

Via CS, Morse HC III, Shearer GM. Autoimmune aspects of HIV infection and relevant murine models. Immunology Today 1990;11:250-255.

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

PROJECT NUMBER

Z01 CB 09285-05 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	IRTA	EIB,NCI
	Jeffrey Richardson	IRTA	EIB,NCI
	John Moriarty	Chemist	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

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

(Formally Titled: "Induction of Class I MHC Gene Expression by Ethanol")

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 recently observed that the 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 ability of cAMP to reduce class I gene expression is not limited to the thyrocyte line. A variety of cells, including lymphoblastoid and fibroblasts, also decrease class I expression in response to cAMP. Although no canonical CRE is found within the 5' upstream region of the class I genes, a series of 5' deletion mutants ligated to the reporter gene, CAT, is being used to functionally localize the cAMP responsive element.

Other agents (including insulin, hydrocortisone, and serum) are capable of modulating class I expression. Serum acts as a negative regulator of class I, both at the cell surface and in steady-state levels of RNA, in a variety of cells. 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). Since the composition of serum is complex and its effects are pleiotropic, the active component in the serum is not known. However, c-jun, which is stimulated following serum treatment, is found to be a negative regulator of class I expression.

Project Description

Major Findings:

MHC class I genes are regulated both by intracellular, tissue-specific signals and extracellular signals. Among the known exogenous regulators are interferon and TNF. Recent studies from our laboratory have identified a number of other extracellular agents and some of the second messengers involved in class I regulation. To examine the effects of hormones on class I expression, we have studied a rat thyrocyte line, FRTL-5, which responds in culture to thyroid stimulating hormone (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 in 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. Because the effects of TSH are transcriptional, we are currently attempting to identify DNA sequence elements involved in the response. A series of 5' deletion mutants, derived from the upstream flanking sequences of a class I gene, ligated to the CAT reporter gene, is being transfected into the FRTL-5 line to map the TSH responsive element. In addition, extracts from FRTL-5 cells cultured in the presence of TSH generate DNA-binding patterns distinct from extracts from untreated cells using DNA segments derived from the 5' upstream region.

Class I regulation by TSH is limited to cells expressing a TSHr. However, a more general response is mediated by fetal calf serum. A variety of cells (including thyrocytes, fibroblasts, 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. Again, a series of 5' deletion mutants, ligated to CAT, has been transfected into fibroblasts to map the DNA response element. In this case, the serum response element is localized within a DNA segment containing a constitutive negative regulatory element (NRE), RE-105. RE-105 has been shown to function as an NRE both in vitro in cell lines and in transgenic animals. However, within RE-105 there is no recognizable serum response element, making it unlikely that SRF is mediating this response. In an attempt to identify transcriptional factor activated by serum, the effect of c-jun on class I expression was examined. Serum is known to activate AP-1, which consists of a heterodimer of jun and fos. Introduction of c-jun into cells reduces class I expression, both of endogenous class I and in cotransfection experiments with a reporter construct. The site of c-jun activity maps to RE-105. Interestingly, c-fos has no effect, either alone or in conjunction with c-jun.

In addition to these factors, other agents (such as insulin, hydrocortisone, and ethanol) have all been found to reduce steady state levels of class I RNA.

Publications: None

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

PROJECT NUMBER
 Z01 CB 09287-04 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Marrow Graft Failure Rejection in All genetic 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:	Kiyoshi Hiruma	Visiting Fellow	EIB, NCI
	Rafael Hirsch	Guest Researcher	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:

1.0

PROFESSIONAL:

0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B, D

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

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system, CTL were cloned from the spleens of sublethally irradiated animals which had rejected MHC disparate marrow grafts. It was found that cloned CTL were sufficient to effect rejection of T cell depleted allogeneic marrow in lethally irradiated animals. The rejection of marrow grafts by CTL was specific for the MHC gene products expressed by the marrow cells and correlated with the cytotoxic specificity of the individual clones. 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. 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 due to suppression of host T cell function and to the release of cytokines associated with in vivo activation of T cells by anti-CD3 antibody.

Project Description

Major Findings:

The purpose of these studies was to directly assess the ability of murine CTL to reject allogeneic marrow grafts and to evaluate the effect that suppression of CTL function in vivo might have on the engraftment of T cell depleted, MHC-mismatched marrow. It was found that CTL clones isolated from 650 cGy sublethally irradiated mice, which had successfully rejected allogeneic marrow, suppressed MHC mismatched marrow graft proliferation (measured by ^{125}I UDR uptake) when adoptively transferred into a 0.25 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 have 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 distinguish death of precursor CTL from induction of anergy. The first difficulty was overcome by incubation of the suppressor cell population with IL-2. Studies with transgenic mice, in which responder T cell populations contain precursor CTL with a defined antigen specificity at high frequency, are in progress in an attempt to overcome the second difficulty noted and define the mechanism by which veto cell activity mediates suppression of CTL responses. Preliminary studies have shown 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.

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 form 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. 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 is in progress.

Publications:

Nakamura H, Gress RE. IL2 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 J, 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, Bluestone JA, Bare CV, Gress RE. Advantages of F(Ab)₂ fragments of anti-CD3 monoclonal antibody as compared to whole antibody as immunosuppressive agents in mice. *Trans. Proc.* 1991;23:270-271.

Hirsch R, Gress RE, Bluestone JA. Anti-CD3-mediated immunotherapy: A murine model. In Burlinghan, W. (ed.): *Critical Evaluation of Antibody Therapies in Transplantation*. CRC Press, Boca Raton. In Press.

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

PROJECT NUMBER

Z01 CB 09288-04 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

Transplantation Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B, D

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

The elimination from marrow of cells expressing T cell surface markers 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. Approaches were developed 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 were used to develop clinical protocols to assess the feasibility of utilizing allogeneic HLA-mismatched, T cell depleted allogeneic marrow and autologous marrow purged of malignant T cells in the treatment of aggressive hematolymphopoietic malignancies. Preclinical studies in rhesus monkeys demonstrated that functional T cell populations are generated in animals receiving T cell depleted autologous marrow. The time course of CD4+ T cell reconstitution and development of in vivo T cell immunocompetence correlated with the number of T cells infused in the marrow, and did not correlate with marrow cell dose or hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of subsequent T cell populations. The mechanisms by which infused, mature T cells generate reconstituting peripheral T cell populations is under investigation in murine models. 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

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 predominant 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 1×10^5 /kg in man. Assays commonly used for the quantitation of residual T cells after T cell depletion are insufficient in sensitivity to detect clinically relevant numbers of residual cells. A limiting dilution assay was therefore developed based on the clonogenic potential of peripheral human T cells; the sensitivity of this assay is sufficient to detect one T cell in 10^5 - 10^6 marrow cells and the specificity has been confirmed by a variety of techniques. This limiting dilution assay has been used to monitor T cell depletion of human marrow. The processing of human marrow for clinical use has now been adapted to a closed, semi-automated system, which includes elutriation followed by treatment with antibody plus complement. The development of reagents and techniques for the removal of cells expressing T cell surface markers from marrow has resulted in clinical trials in both allogeneic marrow (HLA mismatched) and autologous marrow (with removal of malignant T cells) transplantation. With respect to the former, severe GvHD has been prevented with preservation of engraftment. With respect to the latter, the first stage of a phase I study has been completed with definition of a new preparative regimen for the eradication of neoplastic disease in vivo and the development of methods for peripheral marrow progenitor harvest and purging.

To study T cell repertoire generation following T cell depleted marrow transplantation, we characterized the reconstitution 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. These findings are being pursued by molecular analysis of the reconstituting T cell populations and initiation of studies in murine models.

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 T helper cell function has been the scarcity of approaches to evaluate primary, MHC restricted T helper cell responses in man. Studies of human anti-mouse CTL responses indicate that a CD4+ helper pathway functions in the generation of CTL responses and that there exists a dependence on the presence of human antigen presenting cells. Of six xenogeneic responses evaluated, only the human antimurine response was dependent on human antigen presenting cells for CTL generation. The defective human CD4+T helper cell-murine stimulator cell interaction could be bypassed by the addition of exogenous IL-2 indicating that the dependence was at the level of a human helper T cell - stimulator cell interaction and did not reflect requirements at the level of the precursor CTL. The function of the responder antigen presenting cells involved in the human antimurine cytotoxic response was inhibited by chloroquine, suggesting a requirement for antigen processing. Effective presentation of murine stimulating antigen by human antigen presenting cells was completely blocked by anti-human Ia antibody, indicating that the antigen is presented to human T helper cells in association with human class II molecules. These results were consistent with an Ia-dependent recognition of processed murine antigen by human T cells and represents an approach for assessing human T helper cell function and MHC restriction in a primary T cell response. In collaborative studies, CD4+CD8-, CD4-CD8+, and CD3+CD4-CD8- cell lines and clones derived from primary xenogeneic and allogeneic stimulations have been utilized in studies of tyrosine protein kinase signalling and T cell activation.

Publications:

Lucas PJ, Shearer GM, Neudorf S, Gress RE. The human anti-murine xenogeneic cytotoxic response: I. Dependence on responder antigen presenting cells. *J. Immunol.* 1990;144:4548-4554.

Gress RE. Purged autologous bone marrow transplantation in the treatment of acute leukemia. *Oncology* 1990;4:35-43.

Horak ID, Popovic M, Horak EM, Lucas PJ, Gress RE, June CH, Bolen JB. Absence of T-cell tyrosine kinase signalling and calcium mobilization following CD4 association with HIV-1 and HIV-1 gpl20. *Nature* 1990;348:557-560.

Horak ID, Gress RE, Lucas PJ, Horak EM, Leonard WJ, Waldmann TA, Bolen JB. T lymphocyte IL-2 dependent tyrosine protein kinase signal transduction involves the activation of p56lck. *Proc. Natl. Acad. Sci. USA* 1991;88:1996-2000.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09289-02 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

LABORATORY BRANCH

Experimental Immunology Branch

SECTION

Immune Targeting Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

D

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

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.

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 pET11d 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)₃ 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 (gly₄ 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 cysteines 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 cysteines 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 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

Bispecific antibodies can greatly enhance the ability of an antigen to evoke an antibody response, in mice, paralleling their ability to enhance antigen processing and presentation in vitro.

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 09291-02 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:

2.0

PROFESSIONAL:

1.2

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

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

B

SUMMARY OF WORK (Use standard 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 been studying the gene encoding the human ζ subunit. Our studies have focused on several areas: the intron/exon organization of the ζ gene; the determination of sites of transcription initiation; the characterization of this gene as a genetic marker; and the characterization of the human η equivalent. As an extension of these findings we have also been studying the relative expression of ζ and η on a message and protein level during thymic development.

Project Description

Major findings:

Our studies have determined that the human ζ gene consists of 8 exons which encode the ζ message. Intron one is greater than 8kB in size. Introns 2-7 range from 0.7 to 3.0 kB. Ribonuclease protection assays demonstrate multiple sites of transcription initiation the most 3' being approximately 66 bases from the initiating ATG. This is significantly closer to the initiating methionine than had been appreciated in the mouse. There is a unique variable number tandem repeat polymorphism (VNTR) located in the 5th intron within 50 bases of the end of exon 5. This VNTR consists of a 36 base pair repeat with an internal 12 base pair repeat. The presence of this VNTR has allowed us to localize the ζ gene relative to other genetic markers.

We have characterized a region at the 3' end of the gene that is highly homologous on a nucleotide level to the murine η exon. Attempts to characterize a transcript encoding this region have failed to reveal evidence of a properly spliced transcript in human T cells. Additionally PCR amplification of reverse transcribed material has also been without positive results. We have begun to analyze human T cells for the presence of η on a protein level by immunoblotting. Our data suggest that a species with characteristics similar to, but somewhat larger than, the murine η exists in human cells. This species has an Mr consistent with that predicted from the splice product. This species is present at low levels and its level appears to increase relative to ζ on activation of normal human PBL's. Further studies are underway to evaluate whether η expression is truly regulated relative to ζ .

Studies in murine cells have suggested a correlation between the presence of η and the ability of these cells to undergo receptor mediated apoptosis. As apoptosis has been suggested as a means of negative selection in the thymus, we have evaluated the relative frequency of ζ and η message from thymocytes from mice of different ages when there is differential susceptibility to anti-receptor antibody mediated apoptosis. Our studies indicate that there is 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.

Publications:

Zacharchuk CM, Mercep M, June CH, Ashwell JD. Thymocyte Susceptibility to Clonal Deletion Varies During Ontogeny: Implications for Neonatal Tolerance. *J. Immunol*, in press, 1991.

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

PROJECT NUMBER

Z01 CB 09292-02 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 which culminate in cellular activation. The ζ subunit of the TCR has several characteristics which 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. Our studies are carried out both in normal human natural killer cells and T cells as well as in the murine T lymphocyte hybridoma 2B4. Studies in 2B4 cells have been concentrated in two areas; systematic mutagenesis of the ζ subunit; and development of a permeabilized cell system for manipulation of the intracellular environment.

Project Description

Major Findings:

Tyrosine phosphorylation of the ζ subunit has been well characterized in T lymphocytes. To determine whether stimulation of natural killer cells via their Fc receptors would result in the same phenomenon natural killer cells were isolated from normal volunteers. These cells were determined to express ζ on their surface co-associated with the FcR111 IgG receptor. Stimulation of these cells by the use of antibody directed against this receptor results in the rapid phosphorylation of ζ in a fashion similar to that seen in T cells. Perturbation of Fc γ R111 by incubation of NK cells with antibody-coated target cells also induced ζ chain phosphorylation. Other stimuli such as phorbol esters or IL-2 did not result in phosphorylation of ζ . These results demonstrate that receptor stimulated phosphorylation of ζ via receptors to which it is coupled can be extended from T cells to natural killer cells.

Tyrosine phosphorylation of the ζ subunit results in a change in its migration under reducing conditions on SDS/PAGE from 16kD 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. In order to dissect 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 tyrosines 123, 142 and 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 loss of all detectable phospho-zeta suggesting that this residue is particularly crucial in the generation and or maintenance of ζ subunits with multiple tyrosine phosphorylations. The functional consequences of these mutations has been assessed by analysis of IL-2 production and phosphoinositol production. No qualitative abnormalities were detected as a result of the mutations. Further studies are currently being planned to assess the role of phospho- ζ by the generation of transgenic animals containing the mutation at residue 111.

To characterize the molecular events which 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 GTP γ S results in an enhancement of receptor stimulated phosphorylation. This suggests that GTP binding proteins may be involved in the modulation of tyrosine kinase and or tyrosine phosphatase activity. Further studies are underway to expand these observations to other cells and to characterize GTP binding proteins that may play a role in this process.

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.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09295-02 E

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

The Role of HIV gp120 in the Immune Response

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: G.M. Shearer Section Chief EIB, NCI

M. Clerici Visiting Fellow EIB, NCI

B. Rellahan IRT Fellow EIB, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.5

PROFESSIONAL:

0.5

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.)

Infection with HIV results in a degree of immunosuppression that is disproportionate to the number of cells infected. The interaction of the gp120 envelope protein of HIV with cell surface CD4 is important in allowing for viral entry into the cell. In addition to its role in allowing HIV to gain entry into the cell, some studies suggest that this molecule in itself may have immunodulatory effects on uninfected clones and tumor lines. Our initial studies suggest that purified gp120 may have inhibitory effects on cellular function in PBLs from normal human donors.

Project Description

Major Findings:

We have investigated the possibility that gp120 would inhibit in vitro-generated human T helper cell immune responses from HIV- negative donors in a pattern similar to that seen in HIV-infected patients. We have found that the pattern of inhibitory effects of gp120 changed in a dose-dependent way such that higher concentrations inhibited T cell function to all stimuli, whereas lower concentrations selectively inhibit HLA self-restricted responses, but left the responses to alloantigens and mitogens intact. Initial studies in CD4+ T cells suggests that treatment with gp120 has dramatic effects on cellular proliferation induced by anti-T cell receptor antibody. The ability of gp120 to inhibit antibody mediated proliferation will allow us to study the biochemical manifestations of gp120 in human CD4+ populations. We have developed conditions whereby fresh CD4 cells can be analyzed for stimulation specific phosphorylation. Initial studies suggest that gp120 has no effect on the baseline level of tyrosine phosphorylation of the T cell antigen receptor. Studies are in progress to determine whether gp120 effects activation mediated tyrosine kinase and serine kinase phosphorylation events.

This project is 100% AIDS related.

Publications: None

SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1990 - June 1991

The Immunology Branch carries out laboratory investigations in basic immunology with particular emphasis on studies of the major histocompatibility complex (MHC) and its role in transplantation in animal models and in man. The major areas of study are as follows: 1) Immunogenetics of the murine MHC; 2) Development of models for the induction of specific tolerance to MHC products; 3) Genetics of the miniature swine MHC; 4) Molecular biology of porcine class II genes; and 5) Studies of bone marrow and organ transplantation in miniature swine. This report summarizes research efforts in each of these areas during the past year, with reference to the individual annual reports in which details can be found.

1. IMMUNOGENETICS OF THE MURINE MHC

A breeding program is carried out for the maintenance of pedigreed inbred and congenic resistant strains of mice and for the development of new recombinant H-2 haplotypes of value for structural and functional studies of genes within the MHC (5021). This program also includes the production of monoclonal antibodies reactive with products of MHC genes. New strains bearing recombinant MHC haplotypes (of which 22 have been derived from the breeding program of the Immunology Branch) are utilized for the production of new reagents and probes to study the fine structure of the MHC. Because of financial constraints, the backcrossing program by which congenics had been maintained in the past had to be discontinued last year.

2. DEVELOPMENT OF MODELS FOR THE INDUCTION OF SPECIFIC TOLERANCE TO MURINE MHC PRODUCTS

The induction of intentional mixed chimerism across MHC barriers has been investigated as a means of producing specific transplant tolerance (5021). The use of mixtures of T-cell depleted syngeneic plus allogeneic bone marrow has been found to produce permanent, specific tolerance with full immunocompetence. Reconstitution of irradiated animals with a mixture containing T cell depleted syngeneic marrow plus non-T-cell depleted allogeneic marrow has been found to produce complete allogeneic chimerism while avoiding GVHD. This model has been found to have potential usefulness in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect.

In order to decrease the toxicity of ablative radiation regimens used to prepare radiation chimeras, the use of monoclonal anti-T-cell subset antibodies as a substitute for part of the irradiation has been explored. Treatment with such antibodies *in vivo* has been found to deplete mature T-cells effectively from all peripheral lymphoid tissues, but not from the thymus. Selective thymic irradiation has therefore been added to this regimen, and has produced long-term mixed chimerism and specific tolerance across an MHC barrier with a non-lethal preparative regimen. This regimen has now been successfully applied to rat-mouse xenogeneic chimeras as well. In addition, studies of natural antibodies which may be responsible for preventing xenogeneic bone marrow reconstitution have been undertaken. Natural antibodies in mouse serum reactive with rat bone marrow cells have been detected. Correlations between the presence of such antibodies in the serum and graft rejection have been established. These results may explain the requirement for much higher numbers of rat bone marrow cells than allogeneic bone marrow cells to obtain engraftment.

A new protocol has been developed which permits allogeneic engraftment of MHC mismatched bone marrow and spleen cells while avoiding lethal GVHD. The regimen involves administration of IL-2 to recipients for the first three to five days after bone marrow transplantation. The effect is additive to that

of T-cell depleted syngeneic marrow previously described, and avoids both acute and chronic GVHD. A graft versus leukemia (GVL) effect was still seen in animals treated by this new protocol. Since the principal investigator in this research program left the NIH in December 1990, the program was terminated at this date.

3. GENETICS OF THE MINIATURE SWINE MHC

Partially inbred strains of miniature swine, NIH mini-pigs, have been developed over the past fifteen years by this laboratory as a large animal pre-clinical model for transplantation studies (5023). Four new recombinant haplotypes have been identified within these herds, and three of these have been bred to homozygosity. By serologic, biochemical and genetic data all of these recombinants appear to have split class I from class II loci.

Using the new recombinant MHC haplotypes, the relative importance of class I versus class II antigens for allograft survival has been examined. Both class I and class II antigens were found sufficient to produce prompt rejection of skin. However, class II antigen matching appeared to be of overwhelming importance in determining the outcome of vascular allografts such as kidney transplants (5023). The mechanism of tolerance induced by class I mismatched kidneys has been assessed by culturing cells from the transplants. Graft infiltrating lymphocytes (GILS) have been isolated from such cultures and have been shown to specifically suppress generation of CTL reactions against the donor *in vitro*.

4. MOLECULAR BIOLOGY OF PORCINE CLASS II GENES

Because of the importance of class II antigens to renal allograft survival, a molecular approach to the analysis of class II genes in this species has been initiated (5023). DNA from each of the independent MHC haplotypes has been examined by extensive RFLP analysis using probes for human class II α and β genes. Hybridization with the human DQ α cDNA probe revealed a single fragment in each of the enzymatic digests performed, and show limited but definite polymorphism. Hybridization with the human DR α cDNA probe showed a set of bands clearly distinct from those revealed with DQ α , and showed no polymorphism among the herds. In contrast to the unique hybridization patterns of class II α probes, hybridization with class II β probes showed extensive cross hybridization. Polymorphism between all three haplotypes was apparent in many of the digests. Swine class II probes derived from cDNA have been used to map the class II region by pulsed-field gel electrophoresis. All of the porcine class II genes have been mapped.

A genomic library has been constructed from the SLA^c lymphocyte DNA, and cDNA libraries have been prepared from SLA^c and SLA^d spleen message. These libraries have been screened with human class II cDNA probes, and clones analogous to many of the human class II α and β genes have been identified. Sequences of the cDNA clones have been carried out, as have studies of expression following transformation into mouse cell lines. Retroviral vectors have been prepared containing cDNA of all of the expressed porcine genes, along with constitutive and homologous promoters. Several of these have been shown capable of transducing mouse and pig cell lines, leading to expression. These class II recombinant retroviral vectors have been used to transduce pig and mouse bone marrow cells. Efficient expression has been detected in long term cultures of the infected marrow. *In vivo* reconstitutions of irradiated animals with the manipulated marrow have been successfully achieved in the mouse model. Similar experiments are under investigation in the porcine model.

5. STUDIES OF BONE MARROW AND ORGAN TRANSPLANTATION IN MINIATURE SWINE

Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference have been found to frequently develop specific systemic tolerance. The mechanism of this tolerance has been investigated *in vitro* (5023) and has been found to involve a

specific depletion of class I reactive helper T-cell populations. Also, consistent with a lack of T-cell help is the fact that recipients of class II matched renal allografts which went on to accept such grafts long-term showed a brief rejection crisis at approximately fourteen days during which there was a rise in IgM antibodies to SLA class I antigens, which subsided spontaneously without conversion from IgM to IgG.

Because the response to ablative regimens in miniature swine is much more similar to that of man than is the response of rodents, establishment of a bone marrow transplant regimen in this model has been carried out as pre-clinical step (5023). Ablative radiation regimens have been established similar to those used for HLA identical sibling transplants in man, and have been found satisfactory to permit MHC matched bone marrow allografts. A new regimen with fractionated irradiation has been found necessary to permit successful transplantation of MHC mismatched bone marrow. This regimen has been found not sufficient to permit T-cell depletion bone marrow grafts to be accepted. Non T-cell depleted bone marrow grafts across full MHC barriers were found to produce aggressive lethal GVHD. Addition of T-cell depleted autologous bone marrow to the non T-cell depleted allogeneic inoculum has been attempted and, in some cases, has permitted engraftment while avoiding lethal GVHD. These data may have potential applicability to clinical transplantation. A new protocol utilizing IL-2 administration for the first few days after allogeneic bone marrow transplantation has been undertaken on the basis of results in the murine model. The effects of IL-2 in vivo in miniature swine were found to be similar to the effects to the effects described in patients.

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

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

PROJECT NUMBER

Z01 CB 05021-19 I

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Antigens Determined by the Murine Major Histocompatibility Locus

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

PI:	D. H. Sachs	Chief, Transplantation Biology Section	IB, NCI
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	V. S. Abraham	Biotechnology Fellow	IB, NCI
	I. Aksentjevich	Biotechnology Fellow	IB, NCI

COOPERATING UNITS (# any)

S. L. Epstein, Senior Staff Fellow, National Center for Drugs and Biologics, FDA

LAB/BRANCH

Immunology Branch

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Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.0

PROFESSIONAL:

4.0

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

B

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

Studies are being directed toward understanding the major histocompatibility complex, the structure and function of the products of this complex, and manipulations of immune responses to these products. Current studies include: 1) Characterization of major histocompatibility antigens: Congenic resistant strains, of mice developed, maintained, and used in serologic and immunochemical analyses of the MHC products of the mouse; 2) Studies of monoclonal antibodies to H-2 and Ia antigens: Hybridoma cell lines are produced by fusion of immune mouse spleen cells with mouse myeloma cells. The monoclonal anti-H-2 and anti-Ia antibodies produced by these hybridomas are analyzed by serologic and immunochemical means and are used to further characterize the fine structure of the MHC; 3) Mechanism of tolerance to H-2 and Ia antigens: The humoral and cellular responses of radiation bone marrow chimeras are examined, and the mechanism for maintenance of tolerance in these animals is studied; 4) Mixed allogeneic and xenogeneic chimeras: Irradiated animals are reconstituted with mixtures of T-cell depleted donor and host marrow, and the mechanism of tolerance and of immune responsiveness in these animals is studied in vivo and in vitro; 5) Non-lethal preparative regimens for bone marrow transplantation: Attempts to replace lethal irradiation with monoclonal antibody treatment in vivo are examined, and the immune status of the resulting animals is studied; and 6) Graft-vs-leukemia (GVL) effects of mixed bone marrow reconstitution: The effects of reconstitution with T-cell depleted syngeneic plus non-T-cell depleted allogeneic bone marrow on syngeneic leukemia are examined. Addition of IL-2 to the bone marrow reconstitution regimen for the first few days after transplantation has been found to have a marked anti GVHD effect. This project will terminate as of June 30, 1991.

Project Description

Major Findings: 1) Fusion of spleen cells from mice hyperimmunized against H-2 antigens and boosted 2-3 days before fusion has been found to give satisfactory results in the production of anti-H-2 hybridomas. About 70 stable hybridomas have been so far been produced, most of which detect H-2 of Ia antigens of a variety of haplotypes. Panel testing has indicated that most of these antibodies react with public specifications of the H-2 and Ia antigens, while a few appear to detect /private specificities. Numerous crossreactions have been detected using these monoclonal antibodies, which define a variety of new public H-2 and Ia specificities.

2) Using a new MHC recombinant strain (C3H.KBR) we have detected an unusually high responsiveness to Qa antigens. Hybridomas prepared using appropriately immunized spleen cells from this strain yielded 11 new monoclonal antibodies directed against several epitopes of the Qa antigens. Immunoprecipitation studies using these antibodies indicate that at least two different molecules are detectable. In vivo injection of these antibodies has also been found to have profound effects on circulating T-cells. Two of these antibodies of the same immunoglobulin class but directed toward different epitopes of Qa-2 have been found to have different in vivo T-cell depletion characteristics. In vitro studies show that the antibody causing rapid depletion of T-cells in vivo causes much less cell surface modulation of Qa-2 than does the antibody leading to slow depletion. This difference may indicate that the antibody causing modulation reacts with the site on Qa-2 involved in its physiologic ligand binding.

3) A recombinant which occurred during the backcrossing of A.TH has permitted, for the first time, an analysis of A.TH anti-A.TL reactivity in the absence of a Qa-1 antigen difference. Our results with skin grafting experiments indicate that in the absence of a Qa-1 difference, there is marked prolongation of skin graft survival. These studies suggest that a class II difference alone may be insufficient to cause skin graft rejection, and that the class II antigens may actually function by presenting other antigenic differences (e.g., minor antigens of Qa antigens) to the immune system.

4) Mice reconstituted with mixtures of syngeneic plus either allogeneic or xenogeneic bone marrow have been found to manifest specific hyperactivity to donor type skin grafts at 8 weeks following reconstitution. In the case of mixed allogeneic reconstituted animals, both host and donor lymphoid elements are found in the peripheral circulation, while only host elements can be found in the mixed xenogeneic reconstituted animals. In vitro assays have been performed in order to examine the mechanism of transplantation tolerance in these animals. Evidence for generation of high levels of natural suppressor (NS) cells in the early weeks following grafting has been obtained. Characterization of these suppressor cells by antibody reactivity indicates that they are not T cells. They are derived predominantly from the host component of mixed marrow grafts and may be involved in the elimination of those alloreactive cells which otherwise would cause GVH.

5) Because in vitro assays using spleen cells from mixed chimeras showed maximal suppressive activity at approximately 8 days following radiation and bone marrow reconstitution, delayed administration of non T-cell-depleted allogeneic marrow to animals irradiated and reconstituted with T-cell-depleted syngeneic marrow was performed. This procedure was found to markedly reduce the GVH produced by allogeneic T cells and to result in long-term completely allogeneic chimeras.

6) Elimination of T cells from the syngeneic component, but not the allogeneic component of a mixed bone marrow inoculum has been found to produce completely allogeneic chimeras which are, however, protected from lethal GVHD. By adding the EL4 murine leukemia to the syngeneic component of such a reconstituting inoculum, it has been found that this procedure does not eliminate the strong graft-vs-leukemia (GVL) effect of non-T-cell depleted allogeneic bone marrow. The GVL effect has also been seen

when non-T-cell depleted allogeneic marrow was added as late as 8 days following the T-cell depleted syngeneic marrow plus leukemia.

7) Administration of recombinant human IL-2 to recipient mice for the first three to five days after reconstitution fully allogeneic bone marrow plus spleen cells has been found to have a marked anti GVHD effect. The effect was additive to that obtained with T-cell depleted syngeneic marrow. Timing was extremely important, since a delay of seven days led to aggravation of GVHD by the IL-2 administration. The mechanism of this effect is now under study.

8) Using a mixture of anti-CD4 plus anti-CD8 monoclonal antibodies *in vivo*, transient bone marrow engraftment of allogeneic bone marrow was achieved with a total body irradiation dose as low as 300R. Addition of 700R thymic irradiation to this regimen led to long-term mixed chimerism and allogeneic skin graft tolerance. Addition of anti-Thy.1 and anti-NK1 antibodies to the preparative regimen has permitted extension of this model to rat-mouse xenografts.

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

PROJECT NUMBER

Z01 CB 05023-19 I

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Transplantation Antigens of Swine

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

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LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.0

PROFESSIONAL:

4.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

Three herds of miniature swine, homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for human transplantation; 2) Assessment of the immunologic parameters involved in tolerance to allografts in this species; 3) Detection and characterization of intra-MHC recombinants have been obtained and have been or are being bred to homozygosity. Kidney transplants utilizing these new recombinants have shown that selective matching for Class II antigens frequently permits long-term kidney graft survival across a Class I difference; 4) Bone Marrow transplants in miniature swine: the effect of mixing autologous plus allogeneic marrow in the reconstituting inoculum are being examined. This modality is being assessed as a specific preparative regimen for allogeneic organ transplantation; 5) Production and characterization of monoclonal antibodies reactive with subsets of pig lymphocytes: antibodies corresponding to many of the OKT series in man have been identified (including T4, T8 and T11). The effects of these antibodies on in vitro and in vivo transplantation immunity are being assessed, and they are also being used to assess mechanism of tolerance; and 6) Analysis of class II MHC genes: Southern blot analyses using cDNA probes from human class II genes have been performed and indicate that genes corresponding to each of the major human class II loci are present in the pig genome. In addition, a genomic library and two cDNA libraries have been constructed and screened with these probes. Class II genes from the pig herds have been isolated and characterized by hybridization and by sequence analysis. These genes have also been used in vitro and in vivo for transfection studies, and have been incorporated into retroviral constructs for transduction of bone marrow cells. This project will terminate as of June, 30, 1991.

Project Description

Major Findings: 1) Breeding of further generations has continued to be successful. We therefore now have three different herds of swine, each homozygous for a different SLA antigen, as well as five lines bearing intra-MHC recombinants.

2) Transplants of kidneys between recombinant and non-recombinant haplotypes have permitted an evaluation of the effects of selective class I and/or class II matching on vascular allograft survival. A non-MHC-linked gene determining immune responsiveness has been detected and determined to control rejection of vascular grafts across non-MHC differences. In animals bearing the non-rejector gene at this locus, vascular grafts are accepted across major differences and also across class I plus minor differences, but not across the class II differences. These studies indicate, therefore, that class II matching is of major importance for survival of vascular grafts.

3) Skin grafts are promptly rejected across class I and class II differences. However, following successful kidney transplants in the class II matched situation, donor skin grafts are specifically prolonged, indicating that acceptance of the vascular graft induces systemic tolerance.

4) The mechanism of systemic tolerance in class II matched kidney allografts has been examined. Studies on the generation of CTLs *in vitro* indicate that such tolerant animals cannot generate a CTL response across a class I only difference, as can their non-grafted counterparts. The defect appears to involve helper independent class I reactive CTL's, since addition of other helper cells (such as anti-class II reactive populations) leads to normal anti-class I CTL activity. The role of local suppressor activities is suggested by studies of graft infiltrating lymphocytes.

5) Southern blot analyses of lymphocyte DNA from each of the miniature swine herds and recombinant lines have been performed. The data indicate that swine possess a single class II α chain gene corresponding to that of the human DR α chain, DQ α chain, and DP α chain. Of these, the DQ α chain appears to be the most polymorphic within these herds. Approximately 5-7 class II β chain genes have been revealed by these analyses, which show both polymorphism and extensive cross-hybridization between β chains of the three major loci.

6) A complete EMBL-3 phage library has been constructed from the c haplotype and class II genes corresponding to those detected in the Southern blot analysis have been isolated and characterized. Specific hybridizations as well as sequencing data have permitted final identification of each gene.

7) cDNA libraries from the c and d haplotypes have been constructed and screened with human class II probes. cDNA clones corresponding to DR and DQ α and β genes from the SLA^c and SLA^d haplotypes have been isolated. All of these genes have been sequenced and successfully transfected into mouse L cells, leading to cell surface expression of the swine class II antigens on these cell lines.

8) Analysis of the derived amino acid sequence of the SLA DR β^d cDNA gene has led to a surprising finding. Comparison of this sequence with sequences of a panel of human DR β genes indicates that at the amino acid level all three hypervariable regions of the swine sequence are more similar to the corresponding regions of the human DR1 β allele than are the sequences of any other human DR β alleles. At the DNA level, silent substitutions show no differences between different human β alleles. These data therefore suggest a high level of selection leading to the amino acid sequence homology between these species.

9) A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens have been prepared. One of these antibodies recognizes an SLA antigen of the SLA^d haplotype, at least two others react with selective T-cell subpopulations, and at least one antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

10) A protocol which permits bone marrow engraftment across MHC differences has been obtained. Using this protocol, fully allogeneic bone marrow grafts led to lethal GVHD. Using a mixture of T-cell depleted autologous bone marrow plus non-T-cell depleted fully MHC mismatched bone marrow, alloengraftment has been obtained in 17 of 19 animals to date. In 9 of these animals clinical GVHD was self-limited, with resolution by the third week. These data indicate that, as in the murine model, T-cell depleted autologous bone marrow has an anti-GVHD effect when administered as part of the mixed bone marrow reconstitution inoculum. The longest survivor to date has lived 154 days, dying of probable radiation damage to the lungs. On the basis of recent results in the murine model, the use of recombinant IL-2 intravenously for the first three days after bone marrow transplantation has been attempted as a means of preventing lethal GVHD.

11) Retroviral vectors have been constructed incorporating cDNA of swine class II DR α and DR β loci of the SLA^d haplotype along with constitutive promoters. Long-term bone-marrow cultures have been successfully developed for murine as well as for pig marrow. Cells maintained in culture have been transduced and reimplanted in irradiated animals long-term expression of class II cDNA has been observed in mice. These constructs have been effective in transducing both murine and porcine cell lines, leading to expression.

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Annual Report

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY FY 1991

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 three groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma versus normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAbs B72.3 and CC49, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; (b) tissue-associated antigens, such as the breast associated antigen detected by MAb DF3, and the colon-associated antigen detected by MAb D612; and (c) oncogene- or retroviral-related gene products.

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. A serologic map of TAG-72 has been constructed with 19 anti-TAG-72 MAbs. Studies have also been conducted in the analysis of the CC MAbs, COL MAbs, and MAb D612 to define which MAbs are best suited for *in vivo* clinical applications. Emphasis has also been placed on defining and characterizing the tumor associated antigens detected by these MAbs.

Localization and therapy using monoclonal antibodies: Model systems. MAb B72.3, a murine IgG1, has been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, with minimal reactivity to normal adult tissues. B72.3 was therefore chosen as an antibody that could potentially be used for radioimmunodetection and radioimmunotherapy of human carcinomas and is being evaluated in model systems using the LS-174T human colon carcinoma xenograft to determine the feasibility of these studies. A number of new chelate-conjugates have been developed enabling the binding of radiometals to proteins. These new constructs have been covalently linked to B72.3 IgG to determine which resulted in the best biodistribution after labeling with In-111 and Y-88 (used as a substitute for Y-90). Dose fractionalization studies were carried out and demonstrated that splitting the dose of a radiolabeled MAb for therapy can reduce toxicity and increase efficacy. A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain and light chain constant regions.

New anti-TAG-72 MAbs (designated CC) have been developed and compared to B72.3 in the LS-174T model system. All exhibited higher %ID/gm and tumor:tissue ratios than B72.3; differences in the pharmacokinetics were noted among the CC MAbs.

Tumor targeting and pharmacokinetic studies were also carried out using a genetically engineered single chain antigen binding protein (sFv). These studies demonstrated that a relatively small (27kD) single chain molecule can efficiently target a human tumor xenograft.

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 intraperitoneal administration of ^{131}I -B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity is in progress. The use of recombinant/chimeric MAbs has also begun and the use of second and third generation MAb-isotope conjugates are planned.

Augmentation of tumor antigen expression. Heterogeneous TAG-72 and CEA expression has been observed in a variety of human carcinomas. The presence of antigen negative tumor cells is an important consideration when designing clinical protocols which rely on monoclonal antibody binding for either diagnosis or therapy. We identified agents capable of altering cellular differentiation which can upregulate the expression of tumor antigens. We are studying the regulation of CEA expression in a variety of human colon carcinoma cells following interferon treatment. The most dramatic increase in CEA expression and related mRNA transcripts was observed in moderately differentiated colon tumor cells. $\text{IFN-}\gamma$ also increases CEA secretion from highly differentiated colon tumor cells without any dramatic increase in cell-associated CEA expression. Serum samples from patients diagnosed with different adenocarcinomas and treated with recombinant interferon were analyzed retrospectively for changes in circulating TAG-72 and CEA levels. The results indicate that interferon treatment can substantially increase circulating blood levels of TAG-72 and/or CEA shed in approximately 65% of the cases. Furthermore, interferon does not cause the appearance of either tumor antigen in the serum of patients diagnosed with nonadenocarcinomas. These preliminary results indicate that interferon may be an important addition for more sensitive serum assays for the detection of these human tumor antigens. Several human colon carcinoma cell types do not express higher levels of CEA following interferon treatment. However, $\text{IFN-}\gamma$ treatment does induce higher levels of 2'-5'-oligoadenylate synthetase [2'-5' (A)] activity indicating the existence of separate pathways for antigen expression and 2'-5'(A) activity. In addition, treatment of those same cells with analogues of cyclic AMP did increase CEA as well as CEA-related mRNA transcripts. Thus, multiple cellular pathways exist by which differentiation-inducing agents, such as interferon and cyclic AMP, can regulate human tumor antigen expression.

Active immunotherapy to human carcinoma associated antigens. Carcinoembryonic antigen (CEA) is a 180,000-dalton glycoprotein expressed on most gastrointestinal carcinomas and many other human carcinoma types. A 2.4-kb cDNA clone, containing the complete coding sequence, was isolated from a human colon tumor cell library and

inserted into a vaccinia virus genome. This newly developed construct was characterized by Southern blotting, DNA hybridization studies and polymerase chain reaction analysis. The CEA gene was stably integrated in the vaccinia virus thymidine kinase gene. The recombinant was efficiently replicated upon serial passages in cell cultures and in animals. The recombinant virus expresses, on the surface of infected cells, a protein product recognized by a monoclonal antibody (COL-1) directed against CEA. Immunization of mice with the vaccinia construct was found to elicit a humoral immune response against CEA. Pilot studies also demonstrated that the administration of the recombinant CEA vaccinia construct was able to greatly reduce the growth in mice of a syngenic murine colon adenocarcinoma which had been transduced with the human CEA gene. The use of this new recombinant CEA vaccinia construct may thus provide an approach in the specific active immunotherapy of human gastrointestinal cancer and other CEA expressing carcinoma types.

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 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). No amplification of CEA-related genes was found in human colon tumor cell lines expressing high levels of CEA; however, it appeared that the CEA gene(s) were relatively hypomethylated in the CEA expressing tumor cells. We have also investigated the mechanisms responsible for up-regulation of CEA expression following the treatment of human colon tumor cell lines with recombinant human interferon gamma (Hu-IFN- γ). The increased binding of a CEA-specific MAb to tumor cells treated with Hu-IFN- γ correlated with increases in the steady-state levels of CEA-specific message.

Recent experiments have been carried out to derive a mouse tumor cell line expressing CEA for use in both in vitro and in vivo studies. A cDNA clone encoding the human CEA gene has been isolated and subcloned into a eukaryotic expression vector. Clones expressing high levels of cell-surface CEA were isolated and preliminary characterization of the gene products has been carried out. Additional experiments will be carried out to determine the in vivo growth characteristics of clones in normal as well as athymic mice.

Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 mucin antigen. Partial cDNA clones encoding mucins expressed in normal colon and colon tumors have recently been isolated.

Antibody directed cellular immunotherapy of human carcinoma. We investigated the role of two anti-human colon tumor antigen monoclonal antibodies (MAb), chimeric B72.3 (mouse/human)(cB72.3) and native murine D612, in directing and augmenting antibody dependent cellular cytotoxicity (ADCC) activity. cB72.3 antibodies have a human IgG1 (γ 1) or IgG4 (γ 4) constant region. The native murine B72.3 (nB72.3) MAb reacts with antigen expressed by many human carcinomas including colon, breast and ovary but not to most normal adult tissues. D612 (IgG 2a, K) reacts with a 48Kd glycoprotein found on the membrane of malignant and benign human gastrointestinal epithelium but not other adult normal or malignant tissues. ADCC activity of cB72.3 was compared to nB72.3. The xenografted OVCAR-3 human ovarian carcinoma ascites were used as a target in the cytotoxic assay. Lytic activity of the cB72.3 (γ 1) MAb with human peripheral blood mononuclear cells (PMNC) was 1.5 to 50 fold greater than that of the

nB72.3 and cB72.3 (γ 4). Exposure of PMNC to interleukin-2 (IL-2) for 24h to generate LAK cells augmented ADCC mediated by the cB72.3 (γ 1) MAb 2 -to 22- fold. Depletion of FcRgIII positive cells in the PMNC markedly reduced the D612 ADCC. We also studied the effect of human rIL-6 (hrIL-6) on ADCC activity mediated by human PMNC. The ability of hrIL-6 to augment ADCC was measured using D612 MAb and colorectal carcinoma targets LS-174T, WiDr, and HT-29. A significant increase in ADCC activity was observed after PMNC were incubated in 100-400 U/ml of hrIL-6. Human rIL-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. This shows that cB72.3 (γ 1) MAb and murine D612 MAb have appreciable ADCC mediating properties and hrIL-6 can augment ADCC activity of human PMNC using MABs to human tumor antigen and human tumor cells as targets. Results obtained from these studies suggest that cB72.3 and D612 should be considered as candidates for immunotherapy of colon cancer. Furthermore, the data also suggest a potential role of IL-6 in combination with anti-cancer antibodies for cancer immunotherapy.

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 milieu 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 and the action of platelet derived growth factor (PDGF) on human breast cancer cell growth in culture. T47D cells have specific PDGF receptors and respond to its growth promoting signal by inducing phosphorylation of a 65Kd protein in the calelectrin family. Lobulo-alveolar development of the mammary gland requires the priming action of both estrogen and progesterone to induce EGF receptors and production of EGF-like growth factors. In concert with insulin, 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.

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. This hypothesis predicts that specific reversal of TM suppression will lead to a reduction in neoplastic characteristics

of cells transformed by modalities that suppress TM expression. To test this prediction, we have used a retroviral expression vector to insert a full-length cDNA encoding human TM1 into the *v-Ki-ras* transformed cell line DT. The technique proved to have been effective in restoring high levels of expression of TM1 mRNA and protein. Anchorage independent growth in semi-solid agar was virtually eliminated in the TM1 expressing cells. Tumorigenesis in athymic mice was also markedly reduced and when tumors did form, they were no longer found to express the 2.0 kB inserted TM1 mRNA. Thus, cells with restored TM1 expression did not produce tumors. These results are strong evidence that TM suppression plays a necessary causal role in the production by the *ras* oncogene of those components of the transformed phenotype that closely correlate with neoplastic potential: anchorage independence and tumorigenesis in athymic mice. Since many other oncogenic modalities induce TM suppression, it is likely that those modalities also depend on this pathway for transformation. TM suppression thus emerges as a major common step in oncogenesis by many agents.

We have continued our studies on the cytosolic phosphoprotein, Prosolin, which was discovered in this laboratory. It is a major cytosolic protein of proliferating human lymphocytes and promyelocytic cells, but is not expressed in resting peripheral blood lymphocytes. We found that prosolin is an important early target of the activities associated with T-cell receptor (TCr) activation, indicating that prosolin phosphorylation serves to transmit the signal generated by TCr occupancy in the proliferating normal lymphocyte. Prosolin phosphorylation induced by TCr activation or by other treatments is invariably followed by rapid, but temporary, down regulation of DNA synthesis in normal proliferating lymphocytes. This may represent a normal regulatory aspect of the immune system. The rapid phosphorylation of prosolin that follows TCr activation is complex, resulting in 4 phosphorylated forms of the molecule containing variable numbers of phosphorylated serine residues. Evidence suggests that more than one protein kinase is involved in this complex phosphorylation event, and that one of these activities may be deficient in T-cell leukemia cells with the result that some of the phosphorylated forms of prosolin do not appear in such cells. We have cloned and sequenced the prosolin cDNA from normal human peripheral blood lymphocytes and have identified various potential phosphorylation sites.

BIOCHEMISTRY OF ONCOGENES SECTION (Dr. David Salomon, Acting Chief)

The major goal of this project is to understand the role of negative regulatory genes in the mechanism(s) of cellular transformation. Specifically, we have sought to identify genes which can suppress the transformed phenotype and/or negatively regulate cell growth. To identify such genes we have developed an expression cloning assay which depends on alteration of the *Ki-ras* transformed phenotype following uptake and expression of specific DNAs. The source of mRNA for the first cDNA library used in our cloning system was a *Ki-ras* revertant cell line, CHP9CJ. This cell line contains two copies of rescuable *Ki-MuSV* and a high level of activated *ras* p21, but is phenotypically flat and is resistant to transformation by a variety of oncogenes including *v-ras*. Transfection of the CHP9CJ library into *Ki-ras* transformed NIH3T3 cells resulted in the selection of approximately 100 phenotypically altered cell lines. To date cDNAs have been recovered from 20 of these lines and tested in a secondary screening assay. The results indicate that 1 of the 20 cDNAs has the property of reducing growth of *Ki-ras* transformed cells in agar. DNA sequence analysis of this cDNA, referred to as *rsp-1*, reveals a novel gene which appears to be highly conserved among mammalian species; translation of the long open reading frame indicates that the predicted protein product contains a highly leucine rich array which shares homology with the regulatory

region of yeast adenylyl cyclase. Another of the recovered cDNAs was demonstrated by sequence analysis to encode a mouse 4.5S RNA gene. The original transfectant containing this cDNA was morphologically nontransformed and was resistant to retransformation. Transfection of the recovered cDNA into Ki-*ras* transformed cells resulted in the isolation of phenotypically "flat" cells at a low frequency. Analysis of the level of this and other small nuclear RNAs in transformed, revertant, and normal NIH3T3 cells revealed 5-10 fold overexpression of the mouse 4.5S RNA in revertant versus normal cells. In addition there was a reduction in the amount of this RNA in *v-ras*, *v-mos* and *v-src* transformed cells compared to normal controls.

ONCOGENETICS SECTION (R. Callahan, Chief)

Mammary tumorigenesis in inbred and feral mice. The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with mouse mammary tumor virus (MMTV) and have been inbred for a high incidence of mammary tumors. MMTV appears to induce tumors by acting as an insertional mutagen that leads to the activation of a previously silent gene or the rearrangement of a normally expressed gene (*int* gene). We have found that the frequency with which different *int* genes are activated in mammary tumors depends on the particular strain of mice. For instance 80% of the C3H mammary tumors contain a viral induced rearrangement of the *int-1* locus, whereas, in BALB/cfC3H mammary tumors the *int-1* gene is rearranged in only 30% of the tumors. This suggests that the inbreeding program has selected for the fixation of a host mutation which somehow compliments the action of *int-1* gene expression during tumor development. We have expanded our studies to include the feral CZECHII mouse strain which lacks endogenous MMTV genomes in its germline but is congenitally infected with exogenous MMTV. Several viral induced preneoplastic hyperplastic outgrowth (HOG) lines have been developed. Three of these have been found to have a viral insertion at *int-1*. A new *int* locus, designated *int-6*, has been found in a fourth HOG line. Many of the HOG lines spontaneously give rise to mammary tumors, and in two independent cases, the mice also contained metastatic lesions in their lungs. The primary tumors frequently contain additional viral insertions over those observed in the particular HOG line. Similarly, the metastatic tumors frequently contain additional viral insertions over those observed in the primary tumor and the particular HOG line. This suggests that we may be able to identify new *int* genes that are associated with the particular stages of malignant progression. In other studies we have shown that the *int-2* gene product can functionally replace bFGF and compete with it for cellular receptors.

The identification and characterization of human genes associated with neoplasia. The etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken a 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 patients prognosis. In previous studies we have detected frequent amplification of the *c-myc*, *int-2*, and *c-erbB2* proto-oncogenes and the frequent loss of heterozygosity (LOH) on chromosomes 1q, 3p, 11p and 13q. Our current results demonstrate that LOH also frequently occurs on chromosomes 1p, 17p, 17q, and 18q. LOH on chromosome 17q has a significant association ($p < 0.02$) with estrogen receptor negative tumors and LOH is associated ($p < 0.04$)

with histopathological grade III tumors. We have also found associations between specific mutations. For instance, one subset of tumors could be defined by the frequent presence of LOH on chromosomes 11p, 17p, and 18q. Another subset of tumors contained LOH on chromosomes 1p, 13q, and 17q. These results suggest that different subsets of mutations, possibly acting in a complimentary way, are a consequence of the heterogeneous nature of the etiologic factors that provide the selective pressure for the clonal outgrowth of cells containing particular mutations during breast carcinogenesis. We have begun to attempt to identify the target genes affected by LOH. On chromosome 17p a likely candidate is the p53 gene. We have found that the remaining allele of p53, in tumors having LOH on 17p, may also contain a point mutation. On chromosome 17q, we have found LOH at the nm23 gene. This gene is tightly linked to the hereditary breast cancer locus.

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). Using 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.

Site-selective cAMP analogs as antineoplastics and chemopreventives. The physiologic role of cAMP in the growth control of a spectrum of human cancer lines, of a variety of cell types, leukemic lines, *v-ras*H oncogene-transformed NIH/3T3 cells, and *v-ras*^{Ki} or TGF α -transformed NRK (normal rat kidney) cells, is demonstrated by the use of site-selective cAMP analogs. These cAMP analogs, which can select either of the two known cAMP binding sites of the cAMP receptor protein, induce potent growth inhibition, phenotypic change, and differentiation of cancer cells (leukemic cells) at micromolar concentrations with no sign of cytotoxicity. The growth inhibition parallels selective modulation of cAMP-dependent protein kinase isozymes, type I *versus* type II, and suppression of cellular proto-oncogene expression. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancers.

Mechanism of cAMP action in growth control and differentiation. A hypothesis has been presented suggesting that two isoforms of cAMP receptor proteins are crucial effectors in tumorigenesis. The evidence in support of this hypothesis shows that: (1) cAMP transduces dual controls, both positive and negative, on cell growth and differentiation. (2) Such dual controls are respectively governed by two isoforms of cAMP receptor proteins, the type I and type II regulatory subunits of cAMP-dependent protein kinase. (3) In normal physiology, the functional balance of these cAMP receptor isoforms is strictly controlled to meet either stimulation or inhibition of cell growth as it is required, whereas such control is lost in cancer cells. (4) Cancer cells can also be made to differentiate and acquire growth control when the functional balance of these intracellular signal transducers of cAMP is restored by the use of site-selective cAMP analogs, antisense strategy, or gene transfer.

Our purpose is to demonstrate that two isoforms of cAMP receptor proteins each respectively govern dual functions of cAMP in cell proliferation and to show the molecular mechanism of the action of these intracellular signal transducing proteins in the control of cell growth and differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 05190-11 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Jeffrey Schlom Patricia Horan Hand Diane Eggenesperger Chen-Feng Qi Sam Zaremba	Chief Chemist Senior Staff Fellow Visiting Fellow Special Volunteer	LTIB, DCBDC, NCI LTIB, DCBDC, NCI LTIB, DCBDC, NCI LTIB, DCBDC, NCI LTIB, DCBDC, NCI
COOPERATING UNITS (if any) A. Thor, Harvard Medical School; F. Gorestein, Vanderbilt Medical School		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Experimental Oncology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 6.9	PROFESSIONAL: 2.4	OTHER 4.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> 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 <i>in vitro</i> diagnosis via serum assays and/or immunohistopathology, <i>in vivo</i> diagnosis such as gamma scanning, and potentially therapy. The MAbs generated can be classified into three groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma versus normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAbs B72.3 and CC49, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; (b) tissue-associated antigens, such as the breast associated antigen detected by MAb DF3, and the colon-associated antigen detected by MAb D612; and (c) oncogene- or retroviral-related gene products. </p> <p> 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 that B72.3 for some clinical applications. A serologic map of TAG-72 has been constructed with 19 anti-TAG-72 MAbs. Studies have also been conducted in the analysis of the CC MAbs, COL MAbs, and MAb D612 to define which MAbs are best suited for <i>in vivo</i> clinical applications. Emphasis has also been placed on defining and characterizing the tumor associated antigens detected by these MAbs. </p> <p> Work previously reported under project numbers Z01 CB 05233 and Z01 CB 09026 have been incorporated into this project. </p>		

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. We have utilized MAb B72.3 and 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. All 19 MAbs produced immune precipitate lines against purified TAG-72 in double immunodiffusion, indicating that each epitope recognized by a single MAb is present at least twice on 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.

Studies have recently been conducted on the isolation and characterization of the secreted TAG-72 antigen directly from effusions of ovarian, colorectal, pancreatic and endometrial carcinoma patients and the comparison of these with TAG-72 derived from the LS-174T colon carcinoma xenograft. The B72.3 reactive agent, TAG-72 was used as immunogen to produce second generation anti-TAG-72 MAbs. One of these second generation MAbs, CC49, had higher affinity than that of B72.3 and was utilized as an affinity reagent in a procedure to purify the TAG-72 present in the serous effusions of carcinoma patients. A three-step purification procedure, utilizing heat extraction, CC49 antibody affinity chromatography, and gel filtration chromatography, resulted in 1000- to 4400-fold purifications of the TAG-72 derived from effusions, as analyzed using a double-determinant radioimmunoassay. Radiolabeled TAG-72 from each of the effusions demonstrated similar high molecular weight bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Similar results from the various effusions were also obtained in Western blotting analyses. Analyses of TAG-72 from the different effusions in radioimmunoassay using five different anti-TAG-72 MAbs revealed that TAG-72 obtained directly from patients with ovarian, colorectal, endometrial, and pancreatic carcinomas and the LS-174T xenograft are highly similar in terms of immunochemical properties and antigenic profile.

As mentioned above, the B72.3-reactive agent, 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. Two of these MABs, CC49 and CC112, were selected for further immunohistochemical characterization. These MABs were tested here against a spectrum of normal, benign and malignant human adult tissues using the avidin-biotin-peroxidase technique and their reactivity was compared with B72.3. Both CC MABs were more reactive than B72.3 against a range of tumors. Extensive testing with MABs CC49 and B72.3 using serial tissue sections demonstrated that both MABs reacted similarly to most normal adult tissues with MAB CC49 reacting stronger to inflammatory colonic tissue. In 35 of 48 (72%) carcinoma biopsies of the gastrointestinal tract, ovary, breast, and lung in which one of the MABs reacted to at least 20% of the cells, CC49 reacted to a greater percentage of carcinoma cells and/or tumor-associated mucin than B72.3. The reciprocal was observed in only 2% of the carcinomas. This study thus provides evidence that these second generation anti-tumor-associated glycoprotein MABs may be more efficient than B72.3 in the further study of human carcinoma cell populations and in the diagnostic and therapeutic procedures presently being pursued with MAB B72.3.

Enzyme-labeled MABs were used in an immunohistochemical, dual-staining study of 10 colon adenocarcinomas. MABs B72.3 and COL-4, reactive with the high molecular weight TAG-72 antigen and carcinoembryonic antigen (CEA), respectively, were labeled with horseradish peroxidase or alkaline phosphatase. Dual staining using the two MABs on a single tissue section (formalin-fixed, paraffin-embedded) showed that greater numbers of carcinoma cells could be detected by using the combination of the two MABs than could be detected by use of either MAB alone. In many tumors, some carcinoma cells reacted with MAB B72.3, some reacted with MAB COL-4, and some cells reacted with both MABs. Only 1 of 10 carcinomas showed >75% reactive cells when stained with each MAB individually. In 9 of 10 cases, however, >75% of cells reacted when the combination of MABs was used.

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-[³⁵S]methionine-, L-[³H]leucine-, and D-[³H]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 radiiodinated 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-[³H]glucosamine-labeled colon carcinoma cells utilizing MABs D612 and GA733 revealed no cross-reactivity between these antibodies. Similarly, competitive binding studies between MABs 17-1A and KS1/4 and MAB D612 revealed no similarity between the epitopes recognized by MAB D612 and MABs 17-1A and KS1/4. MABs D612 and 17-1A were also titrated in immunoperoxidase staining assays on serial frozen sections of normal and malignant colon. MAB D612 showed a higher titer of immunostaining reactivity with both normal and malignant colon than did MAB 17-1A. MAB D612 showed roughly equivalent immunostaining titers against normal and malignant colon; whereas MAB 17-1A showed a higher titer of

immunostaining reactivity against the normal colon tissue than against the malignant colon. 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 phosphatidylinositol glycan. Radioiodination of the D612 antigen in a plasma membrane-enriched cell fraction by the photoactivatable carbene-generating reagent, 3-(trifluoromethyl)-3-(m-[¹²⁵I]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 K_a of approximately $1.3 \times 10^9 M^{-1}$.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER ZOI CB 08226-15 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Hormones and Growth Factors in Development of Mammary Glands and Tumorigenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Barbara Vonderhaar	Research Chemist	LTIB, DCBDC, NCI
Karen Plaut	IRTA Fellow	LTIB, DCBDC, NCI
Rina Das	Visiting Fellow	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 MAN-YEARS: 4.0	PROFESSIONAL: 3.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The mammary gland is a complex organ whose growth and development are controlled by the interaction of a wide variety of hormones and growth factors. These same factors play fundamental roles in the etiology and progression of the cancerous state. The first event in the action of these hormones and growth factors is the interaction with specific cell associated receptors. The availability and activity of each class of receptor is regulated by the ligand which it recognizes as well as the general hormonal/growth factor milieu of the target cell. Our emphasis has been on the interactions of prolactin (Prl), 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 and the action of platelet derived growth factor (PDGF) on human breast cancer cell growth in culture. T47D cells have specific PDGF receptors and respond to its growth promoting signal by inducing phosphorylation of a 65Kd protein in the calelectrin family. Lobulo-alveolar development of the mammary gland requires the priming action of both estrogen and progesterone to induce EGF receptors and production of EGF-like growth factors. In concert with insulin, Prl and glucocorticoids, EGF or α -TGF can promote full lobulo-alveolar development <i>in vitro</i> . 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. Platelet derived growth factor (PDGF) which is found in milk and is produced by human breast cancer cells in culture has been reported to act in breast tissue only in a paracrine manner. However we have now shown that specific, high affinity PDGF receptors are present on the membranes of T47D cells grown in culture in the presence of 0.5% charcoal stripped serum (CSS) or platelet poor plasma (PPP). In addition, the cells respond to the growth stimulating signal of PDGF with a 2 fold increase in cell number within 3 days of culture. The maximal response occurs with 1.25 half-maximal units of PDGF (125ng/ml). Both PDGF-AB and -AA are active; PDGF-BB has no effect on cell growth. Within 7 min. of addition of PDGF to cultures of the T47D cells, specific phosphorylation of a 65Kd protein in the calelectrin family occurs. Thus it appears that PDGF may be an autocrine growth factor for breast epithelial cells as well.

Lobulo-alveolar development of the mammary gland 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 α -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 will be examined on T47D cells and conditions established for regulation of these molecules by α -IFN, β -TGF and hormones such as estrogen, progesterone and thyroid hormones.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09008-10 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
Patricia Horan Hand	Chemist	LTIB, DCBDC, NCI
Diane Milenic	Microbiologist	LTIB, DCBDC, NCI
John Greiner	Expert	LTIB, DCBDC, NCI
Diane Eggensperger	Senior Staff Fellow	LTIB, DCBDC, NCI
Jim Primus	Expert	LTIB, DCBDC, NCI
Syed Kashmiri	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, Genex; Phil Snoy, FDA, Bethesda, MD		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Experimental Oncology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.2	3.4	2.8
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>MAb B72.3, a murine IgG1, has been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, with minimal reactivity to normal adult tissues. B72.3 was, therefore, chosen as an antibody that could potentially be used for radioimmunodetection and radioimmunotherapy of human carcinomas and is being evaluated in model systems using the LS-174T human colon carcinoma xenograft to determine the feasibility of these studies. A number of new chelate-conjugates have been developed enabling the binding of radiometals to proteins. These new constructs have been covalently linked to B72.3 IgG to determine which resulted in the best biodistribution after labeling with In-111 and Y-88 (used as a substitute for Y-90). Dose fractionation studies were carried out and demonstrated that splitting the dose of a radiolabeled MAb for therapy can reduce toxicity and increase efficacy. A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain and light chain constant regions.</p> <p>New anti-TAG-72 MAbs (designated CC) have been developed and compared to B72.3 in the LS-174T model system. All exhibited higher %ID/gm and tumor:tissue ratios than B72.3; differences in the pharmacokinetics were noted among the CC MAbs.</p> <p>Tumor targeting and pharmacokinetic studies were also carried out using a genetically engineered single chain antigen binding protein (sFv). These studies demonstrated that a relatively small (27kD) single chain molecule can efficiently target a human tumor xenograft.</p>		

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.

MAB B72.3 IgG was radiolabeled with ^{131}I and administered to athymic mice bearing the LS-174T human colon adenocarcinoma xenograft to determine if fractionation of MAB dose had any advantage in tumor therapy. In the LS-174T xenograft, only approximately 30%-60% of tumor cells express the B72.3-reactive TAG-72 antigen. The LS-174T xenograft was used to reflect the heterogeneity of the TAG-72 antigen often seen in biopsy specimens from patients. In contrast to a single 600 μCi dose of ^{131}I -B72.3 IgG where 60% of the animals died from toxic effects, two 300 μCi doses of ^{131}I -B72.3 IgG (total of 600 μCi) reduced or eliminated tumor growth in 90% of mice, with only 10% of the animals dying from toxic effects. Dose fractionation even permitted escalation of the dose to three doses (each 1 wk apart) of 300 μCi of ^{131}I -B72.3 IgG (for a total of 900 μCi) resulting in even more extensive tumor reduction or elimination and minimal toxic effects. The use of an isotype-matched control MAB revealed a nonspecific component to tumor growth retardation, but the use of the specific B72.3 IgG demonstrated a much greater therapeutic effect. Tumors that had escaped MAB therapy were analyzed for expression of the B72.3-reactive TAG-72 antigen with the use of the immunoperoxidase method; they were shown to have the same antigenic phenotype as the untreated tumors. We verified tumor elimination by killing the test animals after a 7-week observation period and performing histologic examination of tumor sites. We also monitored toxic effects by histologic examination of numerous organs, including bone marrow. These studies thus demonstrate the advantage of dose fractionation of a radiolabeled MAB for tumor therapy. We anticipate that the concept of dose fractionation can be practically applied in radioimmunotherapeutic clinical trials with the development and use of recombinant-chimeric MABs and modified constructs.

Biodistribution for five different backbone-substituted derivatives 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}In . 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.

Chimeric mouse/human B72.3 (cB72.3) antibodies having a human IgG1 (γ 1) or IgG4 (γ 4) constant region were compared to the native murine IgG1 B72.3 (nB72.3) MAB for their ability to participate with human effector cells in antibody-dependent cellular cytotoxicity (ADCC). Because the TAG-72 antigen recognized by B72.3 is poorly expressed on tissue-cultured tumor cell lines, the xenografted OVCAR-3 human ovarian carcinoma ascites was used as a cytotoxicity target. The lytic activity of the cB72.3(γ 1) MAB with peripheral blood lymphocytes was 1.5- to 50-fold greater than that of the nB72.3 MAB and usually the cB72.3(γ 4) MAB. However, lymphocytes from some donors had similar ADCC activity with either the cB72.3(γ 1) or cB72.3(γ 4) MAB. The cB72.3(γ 1) and the murine anti-colon carcinoma CO17-1A MAB had comparable activity in mediating ADCC against the OVCAR-3 tumor. Exposure of lymphoid cells to interleukin-2 (IL-2) (100-500 U/ml) for 24 h to generate lymphokine-activated killer (LAK) cells augmented ADCC mediated by the cB72.3(γ 1) MAB 2- to 22-fold. By contrast, LAK cells from most donors expressed weak non-specific cytotoxicity against OVCAR-3 ascites tumor cells. The cB72.3(γ 1), and to a lesser extent, the cB72.3(γ 4) chimera also participated with monocytes in mediating ADCC, but the antibody-dependent lytic potency of monocytic effectors was much weaker than that of IL-2-activated lymphoid cells. These studies show that the cB72.3(γ 1) MAB has appreciable ADCC-mediating properties, suggesting a potential role for its incorporation into treatment strategies utilizing adoptive killer cell and/or lymphokine therapy.

We have carried out the first *in vivo* targeting of tumors with a single-chain antigen-binding protein or sFv. The molecule, which was constructed and expressed in *E. coli*, is a novel recombinant protein composed of variable light-chain (V_L), amino acid sequence of an immunoglobulin tethered to a variable heavy-chain (V_H) sequence by a designed peptide. Despite its rapid clearance, the single-chain antigen-binding protein showed uptake in a human tumor xenograft comparable to that of the Fab' fragment, resulting in tumor to normal tissue ratios comparable to or greater than those obtained with Fab' fragment. These studies thus demonstrate the *in vivo* stability of recombinant single-chain antigen-binding proteins and their potential in some diagnostic and therapeutic clinical applications in cancer and other diseases.

Publications

Schlom, J, Molinolo, A, Simpson, JF, Siler, K, Roselli, M, Hinkle, G, Houchens, DP, and Colcher, D. Advantage of dose fractionation in monoclonal antibody-targeted radioimmunotherapy, *J Natl Cancer Inst* 1990; 82:763-771.

Primus, FJ, Pendurthi, TK, Hutzell, P, Kashmiri, S, Slavin, D, Callahan, R, and Schlom, J. Chimeric B72.3 mouse human (γ 1) antibody directs the lysis of tumor cells by lymphokine-activated killer cells, *Cancer Immunol Immunother* 1990;31:349-357.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09009-10 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Augmentation of Tumor Antigen Expression		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Jack Greiner	Expert	LTIB, DCBDC, NCI
Claudio Dansky- Ullman	Visiting Fellow	LTIB, DCBDC, NCI
Shinya Shimada	Visiting Fellow	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
COOPERATING UNITS (if any) Dr. S. Pestka, UMDNJ - Robert Wood Johnson Medical School, Piscataway, NJ; Drs. E. Borden, D. Smalley, and D. Goldstein, U. Wisconsin Medical Center, Madison, WI; Dr. Y.S. Cho-Chung, LTIB, NCI -		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Experimental Oncology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 5.7	PROFESSIONAL: 4.4	OTHER: 1.3
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Heterogeneous TAG-72 and CEA expression has been observed in a variety of human carcinomas. The presence of antigen negative tumor cells is an important consideration when designing clinical protocols which rely on monoclonal antibody binding for either diagnosis or therapy. We identified agents capable of altering cellular differentiation which can upregulate the expression of tumor antigens. We are studying the regulation of CEA expression in a variety of human colon carcinoma cells following interferon treatment. The most dramatic increase in CEA expression and related mRNA transcripts was observed in moderately differentiated colon tumor cells. IFN- γ also increases CEA secretion from highly differentiated colon tumor cells without any dramatic increase in cell-associated CEA expression. Serum samples from patients diagnosed with different adenocarcinomas and treated with recombinant interferon were analyzed retrospectively for changes in circulating TAG-72 and CEA levels. The results indicate that interferon treatment can substantially increase circulating blood levels of TAG-72 and/or CEA in approximately 65% of the cases. Furthermore, interferon does not cause the appearance of either tumor antigen in the serum of patients diagnosed with nonadenocarcinomas. These preliminary results indicate that interferon may be an important addition for more sensitive serum assays for the detection of these human tumor antigens. Several human colon carcinoma cell types do not express higher levels of CEA following interferon treatment. However, IFN- γ treatment does induce higher levels of 2'-5'-oligoadenylate synthetase [2'-5' (A)] activity indicating the existence of separate pathways for antigen expression and 2'-5' (A) activity. In addition, treatment of those same cells with analogues of cyclic AMP did increase CEA as well as CEA-related mRNA transcripts. Thus, multiple cellular pathways exist by which differentiation-inducing agents, such as interferon and cyclic AMP, can regulate human tumor antigen expression.		

B

Major Findings

The regulation of carcinoembryonic antigen (CEA) expression by recombinant human interferon- γ (IFN- γ) was studied in a series of 7 human colorectal tumor cell lines at various stages of differentiation. Two of the colorectal cell lines were poorly differentiated and did not constitutively express CEA. IFN- γ treatment, however, induced CEA expression in one of those lines (i.e., DLD-1) as evidenced by the appearance of CEA-related mRNA transcripts, as well as the cell surface expression of the antigen as measured by flow cytometry and radioimmunoassay. In the highly differentiated colorectal tumor cell lines, IFN- γ treatment resulted in no detectable change in CEA content in whole cell extracts or in the percentage of cells positive for cell surface CEA expression. In fact, IFN- γ treatment of the highly differentiated LS-174T cell line not only failed to alter CEA expression, but also failed to induce class II human leukocyte antigen (HLA) expression. Therefore, the highly differentiated LS-174T cell line and the poorly differentiated MIP cell line represent colorectal tumor cell types that are unresponsive to the ability of IFN- γ to induce alterations in tumor (i.e., CEA) or normal (i.e., class I and class II HLA) surface antigen expression. The most responsive of human colorectal tumor cells to the ability of IFN- γ to alter CEA expression were the moderately differentiated cells (i.e., HT-29, WiDr, etc.). IFN- γ treatment of those cell types increased the CEA content in cell extracts by 300-400%, and increased the percentage of cells positive for surface CEA expression from 30-45% to >80%. The effect of IFN- γ treatment on 2', 5'-oligoadenylate synthetase (2'-5'A) activity was also studied using 4 of the 7 colorectal cell lines. Constitutive 2'-5'A activity varied approximately 14-fold and was not correlated with degree of cellular differentiation. IFN- γ treatment increased 2'-5'A activity in all 4 colorectal tumor cells tested. In particular, the ability to enhance 2'-5'A activity in the MIP and LS-174T cells, 2 colorectal tumor cell types that previously were shown to be unresponsive to IFN- γ -mediated changes in their antigenic phenotype, clearly separates cellular events regulating 2'-5'A activity from those involved in regulating cell surface antigen expression. The findings also suggested that the regulation of CEA expression by IFN- γ is not related to the degree of cellular differentiation and, furthermore, provide some insight into which human tumor cell populations may be the most amenable to tumor antigen augmentation by IFN- γ in an adjuvant setting with a monoclonal antibody.

IFN- α (A), - β_{ser} , and - γ were analyzed for their relative abilities to increase carcinoembryonic antigen (CEA) expression on, and secretion from, seven different human colorectal tumor cells. LS-174T, CBS and GEO constitutively express high levels of membrane-associated CEA, as well as cytoplasmic CEA, and secrete varying quantities of the antigen. In comparison, GEO cells secrete approximately 3.5- and 10.0-fold higher amounts of CEA than the CBS and LS-174T cells, respectively. Moreover, interferon treatment of GEO cells increased the amount of CEA shed in a dose- and time-dependent manner. The addition of 20-2,000 antiviral units of IFN- α (A), IFN- β_{ser} , or IFN- γ resulted in a dose-dependent increase in cell surface CEA expression as well as the amount of CEA secreted by a moderately differentiated colorectal tumor cell (WiDr). Interferon treatment also increase the level of cell-associated CEA in other human colorectal tumor cells (i.e., HT-29, DLD-1, and CBS); however, the amount shed remained unchanged. Human carcinoma cells that were isolated from malignant effusions and incubated in the presence of IFN- α (A), IFN- β_{ser} , or IFN- γ released increased levels of CEA and/or a second human tumor antigen, tumor-associated glycoprotein-72 (TAG-72). The findings indicate that treatment with either type I or type II interferons can increase the amount

of CEA and/or TAG-72 which is secreted by the tumor cell. Further studies are needed to investigate whether interferon-mediated increases in shedding of a human tumor marker in patients may be important in the diagnostic evaluation of those patients with primary and/or recurrent carcinomas.

In a retrospective study, serum samples from patients entered onto thirteen different clinical protocols and treated with recombinant human interferon were analyzed for changes in the levels of TAG-72 and CEA. Sera were collected from 111 patients diagnosed with adenocarcinoma or nonadenocarcinoma malignancies who received different schedules of interferon (IFN)- γ or IFN β_{ser} alone or in combination. Serum carcinoembryonic antigen (CEA) and tumor-associated glycoprotein-72 (TAG-72) antigen levels were measured to determine whether interferon administration 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 either tumor antigen. Furthermore, interferon administration neither increased TAG-72 or CEA levels nor resulted in the appearance of either tumor antigen in the serum of those patients. In contrast, 59.2% and 75.4% of the patients with adenocarcinoma had positive serum levels of TAG-72 and CEA, respectively, prior to the interferon treatment. IFN- γ and IFN- β_{ser} alone or in combination significantly increased serum TAG-72 or CEA in a high percentage of those patients. For example, in patients with adenocarcinoma who had positive titers of TAG-72 and/or CEA, interferon administration significantly increased the level of either or both antigen(s) in approximately 65% of the cases. 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.

Treatment of human colorectal tumor cells (LS-174T, HT-29 and WiDr) with analogues of cyclic AMP (cAMP) (dibutylryl-cAMP) selectively enhances the expression of CEA. Dose and temporal kinetics results revealed that 8-C1-cAMP was approximately 100-fold more potent than dibutylryl-cAMP for increasing CEA expression. Results demonstrated that 8-C1-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-C1-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-C1-cAMP treatment, suggesting some selectivity for CEA regulation by this cAMP analogue. *In vivo* administration of 8-C1-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 indicated that 8-C1-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-C1-cAMP treatment regulates CEA expression through another cellular pathway which may involve cAMP-dependent protein kinase.

Publications

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Schlom, J, Milenic, DE, Roselli, M, Colcher, D, Bird, R, Johnson, S, Hardman, KD, Guadagni, F, and Greiner, JW. New concepts in monoclonal antibody based radioimmunodiagnosis and radioimmunotherapy of carcinoma. *Nucl Med Biol* 1991;18:425-435.

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

PROJECT NUMBER
 Z01 CB 09018-07 LT1B

PERIOD COVERED
 October 1, 1990 to September 30, 1991

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, laboratory, and institute affiliation)

Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
Diane Milenic	Microbiologist	LTIB, DCBDC, NCI
Diane Eggensperger	Senior Staff Fellow	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
 Dr. J. Carrasquillo, CC, NM; Dr. A. Raubitschek, ROB, DCT, NCI; Dr. E. Reed, COP, DCT, NCI;
 Dr. E. Martin, Ohio State Univ., Dept. Surg.; Dr. S. Larson, Memorial Sloan-Kettering,
 Nucl. Med.; Dr. A. LoBuglio, Univ. of Alabama Medical Center

LAB/BRANCH
 Laboratory of Tumor Immunology and Biology

SECTION
 Experimental Oncology Section

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	2.5	PROFESSIONAL:	1.4	OTHER:	1.1
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CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

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 intraperitoneal administration of 131I-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity is in progress. The use of recombinant/chimeric MAbs has also begun and the use of second and third generation MAb-isotope conjugates are planned.

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 to selectively localize metastatic lesions in 70-80% of the cases. Serum samples from 27 colorectal carcinoma patients who received ^{131}I -B72.3 by i.v. administration were analyzed. Circulating immunoreactive antibody followed a biphasic clearance pattern. High-performance liquid chromatography (HPLC) and SDS-PAGE showed that ^{131}I -B72.3 retained its integrity in the patients' sera. HPLC analysis also demonstrated the presence of immune complexes in the sera of 12 patients; this correlated with elevated levels of circulating TAG-72. Several different HAMA response patterns were detected in the 25 patients' sera that were analyzed; some patients developed HAMA as early as 5-7 days post-MAB injection. Higher doses of administered MAB B72.3 correlated with the development of HAMA ($p=0.007$). The presence of elevated levels of TAG-72 in the patients' pre-inoculum serum was shown to correlate with the detection of lesions by gamma scanning. Serum TAG-72 may serve as criteria for patient selection for immunodiagnostic or immunotherapeutic procedures using MAB B72.3.

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.

The administration of xenogenic MABs leads in many cases to a host immune response represented by the generation of antibodies that can be directed against allotypic, isotypic, and idiotypic determinants present on the xenogenic MAB. Anti-idiotypic antibodies (Ab2s) can be detected by measuring their specific reactivity in sandwich assays using their ability to cross-link labeled Ab1 to Ab1 attached to a solid phase; however, when the MAB used for these studies reacts with a multideterminant tumor-associated antigen found in the circulation (e.g., TAG-72), the utility of these anti-idiotypic assays may be limited. To determine the levels of anti-idiotypic antibodies that could be detected in patients undergoing MAB B72.3-based immunodiagnostic and immunotherapeutic protocols, we investigated the ability of a solid-phase sandwich radioimmunoassay (RIA) to detect anti-idiotypic antibodies raised against B72.3. Furthermore, to overcome the interference of circulating TAG-72 and/or antibodies to allotypic and isotypic determinants in the evaluation of an anti-idiotypic response, we developed a methodology using CC49-coated resin to adsorb the interfering molecules (CC49 is a second generation anti-TAG-72 MAB). Under the conditions established, all of the TAG-72 antigen was removed by adsorption with MAB CC49. Furthermore, since the treatment used an isotype-identical murine MAB, the binding due to the anti-mouse antibodies, other than the anti-idiotypic, was completely abolished after a treatment with

MAB CC49, leaving only the anti-idiotypic component. Analysis of serum samples from patients who had received B72.3 that were positive for human anti-mouse antibodies in the B72.3 sandwich RIA, after the adsorption with CC49 resin, revealed the presence of a B72.3-binding component in 2 of 12 samples. The ability of the adsorbed sera to compete with an anti-B72.3-idiotypic MAB for the binding sites present on the MAB B72.3 confirmed the anti-idiotypic nature of the component being detected in the patient sera.

Serum levels of TAG-72 in patients with carcinoma have been investigated by an immunoradiometric assay (IRMA), CA72-4, utilizing anti-TAG-72 MABs CC49 and B72.3. The cut-off value for CA72-4 assay was determined as 4.0 units/ml according to the mean+2SD (3.9 units/ml), and only 3% of healthy individuals (n=514) demonstrated elevated levels of TAG-72. The average concentration of serum TAG-72 in cancer patients (n=265) was 37.9 units/ml, much higher than that (2.6 units/ml) in patients without malignancy (n=212). Elevated TAG-72 was found in 48% of patients with gastric carcinoma, 52% with colorectal carcinoma, 46% with pancreatic carcinoma, and 60% with ovarian carcinoma. Serum TAG-72 in patients with primary carcinoma was serially measured to determine plasma clearance, and the mean period of decrease to the cut-off value was 23.2 days after removal of the tumors. These results indicate that CA72-4 IRMA may be useful to detect serum TAG-72 antigen in patients with gastrointestinal, ovarian and other epithelial malignancies.

The administration of MABs induces, in many patients, an immunological response represented by the development of human anti-mouse antibodies (HAMA). HAMA have been previously shown to interfere in some assays with the detection of CEA as well as other non-tumor related analytes. The present study was performed to determine whether the CA 72-4 assay is affected by the presence of HAMA, and to establish conditions capable of overcoming this artifact. Serum samples obtained from 8/9 patients entered into a therapeutic protocol using ¹³¹I-labeled MAB B72.3 showed the development of apparently high levels of TAG-72 during the clinical follow-up concurrent with the appearance of elevated titers of HAMA. Heat treatment at 90°C, pH 5.0 in sodium acetate, previously reported as a method of abolishing HAMA interference without affecting CEA levels, resulted in a considerable loss of detectable TAG-72. However, treatment of these samples at 90°C, pH 6.5 in Bis Tris abolished the artifact due to HAMA and resulted in the reversion of reported TAG-72 levels to those observed prior to any MAB administration.

As the use of murine MABs, for both diagnostic and therapeutic applications continues to expand, the identification of this artifactual increase in reported antigen levels due to the development of HAMA has become an important factor in the use of tumor markers, e.g. TAG-72 and CEA, in the follow-up of carcinoma patients.

Publications

Ohuchi, N, Gero, E, Mori, S, Akimoto, M, Matoba, N, Nishihira, T, Hirayamanm K, Colcher, D, and Schlom, J. Clinical evaluation of CA72-4 immunoradiometric assay for serum TAG-72 antigen in patients with carcinoma, *J of Tumor Marker Oncology* 1989; 5:1-10.

Colcher D, Milenic DE, Ferroni, P, Carrasquillo, JA, Reynolds, JC, Roselli, M, Larson, SM, and Schlom, J. *In vivo* fate of monoclonal antibody B72.3 in patients with colorectal cancer, *J Nucl Med*, 1990; 31:1133-1142.

Bell, J, Mojzisek, C, Hinkle, G Jr, Derman, H, Schlom, J, and Martin, E. Intraoperative radioimmunodetection of ovarian cancer using monoclonal antibody B72.3 and a portable gamma detecting probe, *Obstet Gynecol* 1990; 76:607-611.

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Schlom, J, Milenic, DE, Roselli, M, Colcher, D, Bird, R, Johnson, S, Hardman, KD, Guadagni, F, and Greiner, JW. New Concepts in monoclonal antibody based radioimmunodiagnosis and radioimmunotherapy of carcinoma, *Nucl Med Biol* 1991;18:425-435.

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Larson, SM, Carrasquillo, JA, Colcher, D, Yokoyama, Y, Reynolds, JC, Bacharach, SA, Pace, L, Rotman, M, Stabin, M, Neumann, RD, Sugarbaker, P, and Schlom, J. Radiation dosimetry of intraperitoneally administered I-131 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 09021-05 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Isolation and Characterization of Genes Coding for Carcinoma-Associated Antigens		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
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 MAN-YEARS: 3.6	PROFESSIONAL: 0	OTHER: 3.6
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews	B	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>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). No amplification of CEA-related genes was found in human colon tumor cell lines expressing high levels of CEA; however, it appeared that the CEA gene(s) were relatively hypomethylated in the CEA expressing tumor cells. We have also investigated the mechanisms responsible for up-regulation of CEA expression following the treatment of human colon tumor cell lines with recombinant human interferon gamma (Hu-IFN-γ). The increased binding of a CEA-specific MAb to tumor cells treated with Hu-IFN-γ correlated with increases in the steady-state levels of CEA-specific message.</p> <p>Recent experiments have been carried out to derive a mouse tumor cell line expressing CEA for use in both <i>in vitro</i> and <i>in vivo</i> studies. A cDNA clone encoding the human CEA gene has been isolated and subcloned into a eukaryotic expression vector. Clones expressing high levels of cell-surface CEA were isolated and preliminary characterization of the gene products has been carried out. Additional experiments will be carried out to determine the <i>in vivo</i> growth characteristics of clones in normal as well as athymic mice.</p> <p>Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 mucin antigen. Partial cDNA clones encoding mucins expressed in normal colon and colon tumors have recently been isolated.</p>		

Major Findings

Using an assay based on the binding of a CEA-specific MAb, we examined the expression of CEA in human colon tumor and normal fibroblast cell lines. CEA expression was not detectable in the normal fibroblast lines, whereas varying levels of CEA expression were found in the colon tumor cell lines LS-174T, GEO, WiDr, and HT-29. Analysis of the DNA isolated from these cell lines demonstrated that there was no significant deletion or amplification of CEA-related genes in the tumor cell lines. We then used methylation-sensitive enzymes to compare the methylation of genes in the CEA family in normal and tumor cell lines. Our results demonstrate that the CEA genes are relatively hypomethylated in the colon tumor cell lines, and the degree of hypomethylation of these genes appeared to correlate with the level of expression in these cell lines.

The effects of treatment of normal and colon tumor cell lines with recombinant human interferon gamma (Hu-IFN- γ) were then examined. Treatment of two cell lines which express moderate levels of CEA (HT-29 and WiDr) with Hu-IFN- γ resulted in significantly higher levels of antigen expression in these cells. This was found to correlate with increased expression of CEA mRNA in these cells following treatment with the Hu-IFN- γ . Treatment of the LS-174T colon tumor cell line, which expresses high constitutive levels of CEA, with Hu-IFN- γ failed to alter antigen expression or mRNA levels, and no induction of CEA could be seen in the fibroblast cell lines. More recent experiments have been carried out with two colon tumor cell lines, MIP and DLD-1, which constitutively express undetectable levels of CEA. Treatment of DLD-1, but not MIP, with Hu-IFN- γ resulted in the induction of CEA expression. Treatment of HT-29, WiDr, and the normal fibroblast cell lines with Hu-IFN- γ did induce *de novo* expression of the major histocompatibility (MHC) class II antigen DR. These observations suggest that some common factors may be involved in regulation of the CEA and MHC class II genes. In addition, the demonstration that Hu-IFN- γ enhances CEA gene expression in some carcinoma cell lines, and can in addition induce expression in another carcinoma cell line, but fails to induce expression of CEA in fibroblasts supports the notion that Hu-IFN- γ could be used *in vivo* to enhance the tumor targeting of anti-CEA MABs.

We have now begun to develop a mouse model system to study active and passive immunotherapy directed against CEA. For these studies, a mouse tumor cell line (MC-38) which forms tumors in syngeneic C57BL/6 mice was chosen. This cell line was initially transduced with cDNA encoding human CEA, and several clones isolated which express high levels of cell-surface CEA. Initial analysis suggests that some clones express truncated products containing a number of different epitopes present on the normal CEA gene product. These clones will be further characterized, both in terms of their *in vitro* properties and their growth in normal and immunodeficient mice.

A polyclonal antisera has been raised to a synthetic peptide consisting of an amino acid repeat sequence. This sequence was obtained from a purified preparation of TAG-72 antigen isolated from LS-174T colon tumor xenograft. This affinity purified antisera has been shown to react with glycosylated TAG-72 antigen but not with native, non-glycosylated antigen. Thirty clones from a human colon cDNA expression library have been identified with this antisera. They have been classified into 3 categories by their level of reactivity to the antisera. These clones are being subcloned and sequenced. One clone, TAG-21A (800 bp), contains the repeat sequence, but contains no tandem repeats. This clone was used as a nucleic acid probe to screen RNA isolated from a number of human colon tumors. One mucinous human tumor contained large amounts

of TAG-72 mRNA. A new cDNA library was constructed using mRNA isolated from this tumor in order to obtain a full-length clone of TAG-72.

Publications

Robbins, P.F., Kantor, J.A., Salgaller, M., Hand, P.H., Fernsten, P.D., and Schlom, J. Transduction and expression of the human carcinoembryonic antigen (CEA) gene in a murine colon carcinoma cell line, *Cancer Res.* 1991 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09025-04 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
Kwong-Y. Tsang	Expert	LTIB, DCBDC, NCI
F. James Primus	Expert	LTIB, DCBDC, NCI
Margaret Finch	Senior Staff Fellow	LTIB, DCBDC, NCI
Rosaria DeFillippi	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 MAN-YEARS: 3.5	PROFESSIONAL: .2	OTHER: 3.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) We investigated the role of two anti-human colon tumor antigen monoclonal antibodies (MAb), chimeric B72.3 (mouse/human)(cB72.3) and native murine D612, in directing and augmenting antibody dependent cellular cytotoxicity (ADCC) activity. cB72.3 antibodies have a human IgG1 (γ1) or IgG4 (γ4) constant region. The native murine B72.3 (nB72.3) MAb reacts with antigen expressed by many human carcinomas including colon, breast and ovary but not by most normal adult tissues. D612 (IgG2a, K) reacts with a 48Kd glycoprotein found on the membrane of malignant and benign human gastrointestinal epithelium but not other adult normal or malignant tissues. ADCC activity of cB72.3 was compared to nB72.3. The xenografted OVCAR-3 human ovarian carcinoma ascites were used as a target in the cytotoxic assay. Lytic activity of the cB72.3 (γ1) MAb with human peripheral blood mononuclear cells (PMNC) was 1.5 to 50 fold greater than that of the nB72.3 and cB72.3 (γ4). Exposure of PMNC to interleukin-2 (IL-2) for 24h to generate LAK cells augmented ADCC mediated by the cB72.3 (γ1) MAb 2 to 22 fold. Depletion of FcγRIII positive cells in the PMNC markedly reduced the D612 ADCC. We also studied the effect of human rIL-6 (hrIL-6) on ADCC activity mediated by human PMNC. The ability of hrIL-6 to augment ADCC was measured using D612 MAb and colorectal carcinoma targets LS-174T, WiDr, and HT-29. A significant increase in ADCC activity was observed after PMNC were incubated in 100-400 U/ml of hrIL-6. Human rIL-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. This shows that cB72.3 (γ1) MAb and murine D612 MAb have appreciable ADCC mediating properties and hrIL-6 can augment ADCC activity of human PMNC using MAbs to human tumor antigen and human tumor cells as targets. Results obtained from these studies suggest that cB72.3 and D612 should be considered as candidates for immunotherapy of colon cancer. Furthermore, the data also suggest a potential role of IL-6 in combination with anti-cancer antibodies for cancer immunotherapy.		

Major Findings

A monoclonal antibody (MAb), designated D612 (IgG2a), was generated by immunization of mice with a membrane enriched fraction of a moderately differentiated primary colon adenocarcinoma. Using membrane extracts derived from a variety of cell lines and normal and neoplastic tissues, D612 was found to react specifically with 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 1×10^6 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 10U/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.

The ability of the cB72.3 (γ_1) or (γ_4) MAbs to mediated ADCC with human PMNC was investigated in comparison to that of nB72.3 MAb. nB72.3 is a K light chain containing IgG₁ MAb. MAb IgG₁ isotype is not commonly connected with ADCC activity. nB72.3 MAb recognizes TAG-72 antigen which is poorly expressed on tissue culture tumor cells. The xenografted OVCAR-3 human ovarian carcinoma ascites was used as target cells. OVCAR-3 tumor usually has a low sensitivity to non-specific cytotoxicity which was about 2% or less. The nonspecific cytotoxicity increased to about 6% or less when LAK cells were used as effectors. Upon the addition of nB72.3 at 25 μ g/well, modest augmentation of ADCC activity were observed. The cB72.3 (γ_1) MAb was found to mediate much greater ADCC as compared to the nB72.3 MAb. The nB72.3 MAb was 2-4 times less active than the cB72.3 (γ_1) in mediating ADCC with LAK cells. At 4 h assay incubation time of the ADCC, the 17-1A MAb and cB72.3 (γ_1) at low level showed a small amount of ADCC activity after

IL-2 treatment of effector cells but not with unactivated PMNC. By contrast, there was a marked increase in ADCC at 24 h incubation time with both 17-1A and cB72.3 (γ_1) MAb. The increase in ADCC measured at 24 h was accompanied by a much lower increase in nonspecific lysis. IL-2 increased ADCC mediated by the cB72.3 (γ_1) MAb 2 to 22 fold as compared to the combined cytotoxic activity of LAK cell alone and unstimulated effector cells in the presence of antibody. IL-2 augmented ADCC activity mediated by cB72.3 (γ_1) was 1.5-2 times greater than the cytotoxicity mediated by nB72.3. The ADCC mediating properties of the cB72.3 (γ_1) MAb were greater than the cB72.3(γ_4) MAb.

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.

Publications

Primus, FJ, Pendurthi, TK, Hutzell, P, Kashmiri, S, Slavin, DC, Callahan, R, and Schlom J. Chimeric B72.3 mouse/human (IgG1) antibody directs the lysis of tumor cells by lymphokine-activated killer cells, *Cancer Immunol Immunother* 1990;31:349-357.

Pendurthi, TK, Parker, R, Schlom, J, and Primus, FJ. Lymphokine-activated killer cell cytotoxicity against human colon carcinomas enhanced by monoclonal antibody D612, *Int J Cancer* 1990;46:1021-1028.

Tsang, KY, Finch, MD, Primus, FJ, and Schlom, J. Human rIL-6 enhances antibody dependent cellular cytotoxicity of human tumor cells mediated by human peripheral blood mononuclear cells, *Cancer Immunol Immunother* (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09028-01 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
Judy Kantor	Expert	LTIB, DCBDC, NCI
Paul Robbins	Senior Staff Fellow	LTIB, DCBDC, NCI
Anita Chang	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 MAN-YEARS: 4.2	PROFESSIONAL: 2.7	OTHER: 1.5
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Carcinoembryonic antigen (CEA) is a 180,000-dalton glycoprotein expressed on most gastrointestinal carcinomas and many other human carcinoma types. A 2.4-kb cDNA clone, containing the complete coding sequence, was isolated from a human colon tumor cell library and inserted into a vaccinia virus genome. This newly developed construct was characterized by Southern blotting, DNA hybridization studies and polymerase chain reaction analysis. The CEA gene was stably integrated in the vaccinia virus thymidine kinase gene. The recombinant was efficiently replicated upon serial passages in cell cultures and in animals. The recombinant virus expresses, on the surface of infected cells, a protein product recognized by a monoclonal antibody (COL-1) directed against CEA. Immunization of mice with the vaccinia construct was found to elicit a humoral immune response against CEA. Pilot studies also demonstrated that the administration of the recombinant CEA vaccinia construct was able to greatly reduce the growth in mice of a syngenic murine colon adenocarcinoma which had been transduced with the human CEA gene. The use of this new recombinant CEA vaccinia construct may thus provide an 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 pumped 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 Hind III J fragment. DNA hybridization studies were performed to show that the recombinant virus contained the CEA gene. Recombinant and wild type virus were plaque 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 2.1Kb 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 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 specific active immunotherapy protocols. We will compare the efficacy of tumor rejection using recombinant vaccinia virus vaccinations with immunization schedules using purified human CEA protein, CEA specific peptides and anti-idiotypic antibodies. We will define the immunological mechanisms which might be involved using these specific protocols.

Publications

Kaufman, H, Schlom, J, and Kantor, J. A recombinant vaccinia virus expressing human carcinoembryonic antigen (CEA), *Int J Cancer* 1991 (in press).

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

PROJECT NUMBER

Z01 CB 09006-09 LT1B

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Studies on the Nature and Function of the Phosphoprotein, Prosolin

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

Herbert L. Cooper	Chief, Cell & Molec. Physiol. Section	LTIB, DCBDC, NCI
Rebecca Fuldner	Biotechnology Research Fellow	LTIB, DCBDC, NCI LT1B,
Richard Braverman	Chemist	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 MAN-YEARS:

3.0

PROFESSIONAL:

1.5

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

B

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

We have continued our studies on the cytosolic phosphoprotein, Prosolin, which was discovered in this laboratory. It is a major cytosolic protein of proliferating human lymphocytes and promyelocytic cells, but is not expressed in resting peripheral blood lymphocytes. We found that prosolin is an important early target of the activities associated with T-cell receptor (TCr) activation, indicating that prosolin phosphorylation serves to transmit the signal generated by TCr occupancy in the proliferating normal lymphocyte. Prosolin phosphorylation induced by TCr activation or by other treatments is invariably followed by rapid, but temporary, down regulation of DNA synthesis in normal proliferating lymphocytes. This may represent a normal regulatory aspect of the immune system. The rapid phosphorylation of prosolin that follows TCr activation is complex, resulting in 4 phosphorylated forms of the molecule containing variable numbers of phosphorylated serine residues. Evidence suggests that more than one protein kinase is involved in this complex phosphorylation event, and that one of these activities may be deficient in T-cell leukemia cells with the result that some of the phosphorylated forms of prosolin do not appear in such cells. We have cloned and sequenced the prosolin cDNA from normal human peripheral blood lymphocytes and have identified various potential phosphorylation sites.

Major findings

We have continued our studies on the cytosolic phosphoprotein, Prosolin, which was discovered in this laboratory. It is a major cytosolic protein of proliferating human lymphocytes and promyelocytic cells, but is not expressed in resting peripheral blood lymphocytes.

We found that prosolin was rapidly phosphorylated in normal proliferating human lymphocytes by agents which activate the T-cell receptor [TCr] (anti-CD3 MAb; PHA) or by agents which bypass the TCr but directly induce activities that normally occur following TCr activation (phorbol esters; Ca^{2+} ionophores). This demonstrates that prosolin is an important early target of the activities associated with TCr activation, indicating that prosolin phosphorylation serves to transmit the signal generated by TCr occupancy in the proliferating normal lymphocyte.

Prosolin phosphorylation induced by TCr activation or by other treatments is invariably followed by rapid but temporary down regulation of DNA synthesis in normal proliferating lymphocytes. Thus, TCr activation in proliferating lymphocytes, unlike the case in resting lymphocytes, results in a temporary suspension of proliferative activity. This may represent a normal regulatory aspect of the immune system. The different response pattern of resting compared with proliferating lymphocytes may reflect the fact that prosolin is not expressed in resting lymphocytes, and therefore is not available for phosphorylation events which may transmit the growth inhibitory signal.

The rapid phosphorylation of prosolin that follows TCr activation is complex, resulting in four phosphorylated forms of the molecule containing variable numbers of phosphorylated serine residues. Evidence suggests that more than one protein kinase is involved in this complex phosphorylation event, and that one of these activities may be deficient in T-cell leukemia cells with the result that some of the phosphorylated forms of prosolin do not appear in such cells. T-cell leukemia cells also do not display down-regulation of DNA synthesis in response to agents that induce prosolin phosphorylation. This suggests that these leukemic cells are unable to transmit a normal growth regulatory signal (prosolin phosphorylation) that suspends DNA synthesis due to deficiency of a specific protein kinase activity. This defect may play a role in the disordered growth regulation of T-cell leukemia cells.

We have cloned and sequenced the prosolin cDNA from normal human peripheral blood lymphocytes and have identified various potential phosphorylation sites.

Future Plans

Although this project is of great interest and deserving of further work, current strictures on expenditure and laboratory personnel make it necessary to discontinue work in this area for the foreseeable future after experiments and manuscripts currently in progress are completed.

Publications

Cooper, HL, Fuldner, R, McDuffie, E, and Braverman, RA. A specific defect of prosolin phosphorylation in T-cell leukemic lymphoblasts is associated with impaired down-regulation of DNA synthesis, *J Immunol* 1990; 145: 1205-1213.

Cooper, HL, Fuldner, R, McDuffie, E, and Braverman, R. T-cell receptor activation induces rapid phosphorylation of prosolin which mediates down-regulation of DNA synthesis in proliferating peripheral lymphocytes, *J Immunol* In press, 1991.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09022-05 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cytoskeletal Proteins in Oncogenic Transformation and Human Neoplasia		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Herbert L. Cooper Gaddamanugu Prasad Rebecca Fuldner	Chief, Cell. & Molec. Physiol. Section Visiting Fellow Senior Staff Fellow	LTIB, DCBDC, NCI LTIB, DCBDC, NCI 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 MAN-YEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) We have continued our studies on the 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. This hypothesis predicts that specific reversal of TM suppression will lead to a reduction in neoplastic characteristics of cells transformed by modalities that suppress TM expression. To test this prediction, we have used a retroviral expression vector to insert a full-length cDNA encoding human TM1 into the v-Ki-ras transformed cell line DT. The technique proved to have been effective in restoring high levels of expression of TM1 mRNA and protein. Anchorage independent growth in semi-solid agar was virtually eliminated in the TM1 expressing cells. Tumorigenesis in athymic mice was also markedly reduced and when tumors did form, they were found no longer to express the 2.0 kB inserted TM1 mRNA. Thus, cells with restored TM1 expression did not produce tumors. These results are strong evidence that TM suppression plays a necessary causal role in the production by the ras oncogene of those components of the transformed phenotype that closely correlate with neoplastic potential: anchorage independence and tumorigenesis in athymic mice. Since many other oncogenic modalities induce TM suppression, it is likely that those modalities also depend on this pathway for transformation. TM suppression thus emerges as a major common step in oncogenesis by many agents.		
651		

Major Findings

We have continued our studies on the relationship of suppression of tropomyosin (TM) synthesis to neoplastic transformation. Previous work has established that expression of two higher M_r TMs (TM1 and TM2) was consistently reduced or eliminated in murine cells transformed by a variety of retroviral oncogenes, DNA tumor viruses, chemical carcinogens, or transforming growth factors (TGFa). We have also examined six 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.

This hypothesis predicts that specific reversal of TM suppression will lead to a reduction in neoplastic characteristics of cells transformed by modalities that suppress TM expression. To test this prediction, we have inserted a full-length cDNA encoding human TM1 into cell line DT, which is a line of NIH 3T3 cells doubly transformed by *v-Ki-ras* (obtained from Dr. R. Bassin). We have previously shown that DT cells have suppressed synthesis of TM1 and TM2. This was accomplished by means of infection with a retroviral vector. A full length cDNA (TMe1) encoding human TM1 was cloned in this laboratory from the LS-174T colon carcinoma cell line. It was subcloned into the pBNC retroviral vector (obtained from the laboratory of Dr. French Anderson). The vector contains the LTRs and packaging signal (Ψ) of MMLV, but lacks all other components of the retrovirus. It contains an SV40 neo-resistance sequence and a cytomegalovirus transcription promoter, which drives expression of the inserted TMe1 sequence. The construct was transfected into the Ψ -2 ecotropic packaging line where it was integrated and transcribed into RNA which was packaged and secreted as replication-defective infectious particles using viral protein components provided by the Ψ -2 cell. The infectious supernatants of Ψ -2 cultures were used to infect DT cells. Cell clones (S-clones) expressing the vector were obtained by selection for neomycin resistance with G418. Control clones (V-clones) were obtained by using vector with no TMe1 insert.

Of eight S-clones selected, six proved to have high levels of expression of TM1 protein as determined by 2-dimensional electrophoretic mobility, heat stability, and immunoprecipitation. Levels of TM1 expression ranged from 5 to 15 times greater than that of the parent DT cell in the high-expressor S-clones. V-clones showed no alteration in TM1 expression. Northern blot analysis confirmed the presence in the high expressor S-clones of mRNAs containing the inserted TMe1 sequence (2.0 kB, including some vector-derived sequence) as well as the endogenous TM1 sequence (1.1 kB). The 2.0 kB mRNA was absent from V-clones. These results demonstrate that the retroviral vector provides an efficient method of obtaining high level expression of exogenous TM sequences in target cells.

Replacement of TM1 expression had a modest effect on the cell culture morphology of S-clone cells. They no longer formed large, dome-shaped foci as in DT cells or in V-clones, but continued to display reduced contact inhibition (multilayered growth). Cell growth rates were variably affected. However, anchorage independent growth in semi-solid agar was virtually eliminated in the S-clone cells. Tumorigenesis in

athymic mice was also markedly reduced and when tumors did form, they were no longer found to express the 2.0 kB inserted TMe1 mRNA. Thus, cells with restored TM1 expression did not produce tumors.

These results are strong evidence that TM suppression plays a necessary causal role in the production by the *ras* oncogene of those components of the transformed phenotype that closely correlate with neoplastic potential: anchorage independence and tumorigenesis in athymic mice. Since many other oncogenic modalities induce TM suppression, it is likely that those modalities also depend on this pathway for transformation. TM suppression thus emerges as a major common step in oncogenesis by many agents.

Our ability to reverse neoplastic behavior in this system raises the possibility of TM replacement as a future therapeutic modality. That TM suppression is common to many oncogenic modalities raises the possibility that the single therapeutic maneuver of TM replacement may be effective in tumors of diverse origins.

Future Plans

Future plans for this project include development of new retroviral vectors for expression of TMs in human cells; cloning and expression of TM2 in DT cells; 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

Yanagihara, K., Ciardiello, F., Talbot, N., McGready, M., Cooper, H.L., Benade, L., Salomon, D.S., and Bassin, R.H. Isolation of a new class of "flat" revertants from *ras*-transformed NIH 3T3 cells using *cis*-4-hydroxy-L-proline, *Oncogene* 1990; 5: 1179-86.

Prasad, G.L., Meissner, S., Sheer, D.G., and Cooper, H.L. A cDNA encoding a muscle-type tropomyosin cloned from a human epithelial cell line: Identity with human fibroblast tropomyosin, TM1. *Biochem. Biophys. Acta*, In press, 1991.

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

PROJECT NUMBER
 Z01 CB 04848-19 LTIB

PERIOD COVERED
 October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 "Anti-Oncogenes": The Analysis of Cellular Resistance to Transformation

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

Robert Bassin	Chief, Biochemistry of Oncogenes	LTIB, DCBDC, NCI
Mary Lou McGeady Cutler	Section	LTIB, DCBDC, NCI
Neil Talbot	Expert	LTIB, DCBDC, NCI
Lorenzo Zanoni	Guest Worker	LTIB, DCBDC, NCI

COOPERATING UNITS (if any)
 Dr. Makoto Noda, Riken Institute of Science, Japan

LAB/BRANCH
 Laboratory of Tumor Immunology and Biology

SECTION
 Biochemistry of Oncogenes

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	4.2	PROFESSIONAL:	3.0	OTHER:	1.2
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CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

B

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

We have constructed a cDNA library from CHP9CJ cells, a v-Ki-ras revertant cell line, in a eukaryotic expression vector. Using an expression cloning strategy that includes transfection into Ki-ras transformed cells and selection for drug resistance and phenotypic change, we have isolated two candidate cDNA molecules which alter the transformed phenotype. One cDNA encodes a mouse 4.5S gene, a small nuclear RNA that is highly overexpressed in ras-revertant cell lines. The other is a novel gene, referred to as *rsp-1* which contains, as part of its long open reading frame a leucine repeat array homologous to the leucine arrays in the regulatory region of yeast adenyllylcyase. Our current efforts are aimed at determining the functions of these genes in normal and transformed cells.

Major Findings

The major goal of this project is to understand the role of negative regulatory genes in the mechanism(s) of cellular transformation. Specifically, we have sought to identify genes which can suppress the transformed phenotype and/or negatively regulate cell growth. To identify such genes, we have developed an expression cloning assay which depends on alteration of the *Ki-ras* transformed phenotype following uptake and expression of specific cDNAs. The isolation of *k-rev-1* by Dr. Makoto Noda has demonstrated this to be an effective approach for the isolation of transformation suppressor genes. The source of mRNA for the first cDNA library used in our cloning system was a *Ki-ras* revertant cell line, CHP9CJ, isolated in this laboratory. This cell line contains two copies of rescuable *Ki-MuSV* and a high level of activated *ras* p21, but is phenotypically flat and resistant to transformation by a variety of oncogenes including *v-ras*. The analysis of the CHP9CJ cell line suggested that it contained an alteration affecting the *v-ras* transformation pathway but not the *c-ras* signalling pathway. In addition, this cell line has a longer doubling time than NIH3T3 cells. Therefore, the use of this cell line as a source of material for cDNA library construction increased the potential for isolation of cDNAs which negatively regulate growth in normal cells as well as cDNAs which negatively regulate pathways unique to the transformed cell.

The CHP9CJ library was constructed in a novel vector, p521, which allowed directional cloning of the cDNA and provided drug resistance markers for both prokaryotic and eukaryotic cells. Following transfection of the CHP9CJ cDNA library into *Ki-ras* transformed NIH3T3 cells, selection for drug resistance and treatment to preferentially remove transformed cells, approximately 100 phenotypically altered cell lines were isolated. To date cDNAs have been recovered from 20 of these lines and tested in a secondary screening assay. The results indicate that 1 of the 20 cDNAs has the property of reducing growth of *Ki-ras* transformed cells in agar. DNA sequence analysis of this cDNA, referred to as *rsp-1*, reveals a novel gene which appears to be highly conserved among mammalian species; translation of the long open reading frame indicates that the predicted protein product contains a highly leucine rich array which shares homology with the regulatory region of yeast adenylyl cyclase. Our current studies are aimed at determining the function of this molecule in normal and transformed cells.

Another of the recovered cDNAs was demonstrated by sequence analysis to encode a mouse 4.5S RNA. The original transfectant containing this cDNA was morphologically nontransformed and was resistant to retransformation by *v-ras*, *v-mos*, and *v-src*. Transfection of the recovered cDNA into *Ki-ras* transformed cells resulted in the isolation of phenotypically "flat" cells at a low frequency. Analysis of the level of this and other small nuclear RNAs in transformed, revertant, and normal NIH3T3 cells revealed 5-10 fold overexpression of the mouse 4.5S RNA in revertant versus normal cells. In addition, there was a reduction in the amount of this RNA in *v-ras*, *v-mos* and *v-src* transformed cells compared to normal controls. The increase in the RNA level in the revertant cell lines could be attributed to both transcriptional and post-transcriptional events while the reduction in the transformed cells was entirely a result of the rate of transcription. Analysis of the level of other small nuclear RNAs in the transformed and revertant cell lines revealed that only the mouse B-2 repeat sequences share this unusual transcriptional pattern. Our current efforts are aimed at determining the contribution of these small nuclear RNAs to the maintenance of the nontransformed phenotype.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 09003-09 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
David S. Salomon	Acting Chief, Biochemistry of Oncogenes Section	LTIB, DCBDC, NCI
Toshiaki Saeki	Visting Fellow	LTIB, DCBDC, NCI
Nicola Normanno	Visting Fellow	LTIB, DCBDC, NCI
COOPERATING UNITS (if any) Dr. Robert Callahan, Chief, Oncogenetics Section, LTIB, NCI; 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 Biochemistry of Oncogenes Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 3.7	PROFESSIONAL: 2.7	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.) Transforming growth factor α (TGF α), amphiregulin (AR) and cripto are proteins that are structurally and in some cases functionally related to epidermal growth factor (EGF) in that TGF α and AR can bind to the EGF receptor. TGF α 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 TGF α 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 <i>c-Ha-ras</i> protooncogene, but not with a <i>c-erbB-2</i> oncogene, results in an increase in TGF α expression. Also, overexpression of human TGF α cDNA in these cells leads to <i>in vitro</i> transformation. Addition of an anti-EGF receptor blocking antibody or an anti-TGF α neutralizing antibody can partially or completely inhibit the growth of the <i>ras</i> or TGF α transformed mammary cells, suggesting the establishment of an external autocrine loop. Estrogens can increase TGF α 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 TGF α 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 TGF α promoter contains a <i>cis</i> -acting estrogen-responsive element (ERE) MCF-7 cells were infected with a recombinant amphotropic TGF α antisense expression vector. Expression of this antisense RNA leads to partial reduction in basal and estrogen-induced TGF α 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 TGF α 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 TGF α mRNA expression and TGF α protein production suggesting that TGF α 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 TGF α 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 TGF α . These TGF α 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 TGF α transformed cells in soft agar can be inhibited with either an anti-EGF receptor blocking antibody or with an anti-TGF α neutralizing antibody demonstrating that TGF α 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 TGF α 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 TGF α transformed cells to respond to lactogenic hormones suggesting that secreted TGF α 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 TGF α and of the *erb* B-2 receptor by an unidentified ligand(s) has different effects upon mammary epithelial cell differentiation. TGF α 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, TGF α or basic FGF. Likewise, co-cultivation 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 TGF α and basic FGF and the fibroblasts express basic FGF mRNA.

The expression of TGF α mRNA and TGF α 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 17 β -estradiol (E2) whereas in ER negative breast cancer cell lines such as MDA-MB-231 cells basal levels of TGF α are generally higher than in the ER positive cell lines and insensitive to E2 regulation. To ascertain if E2 can directly regulate TGF α expression through the TGF α promoter, MCF-7 and ZR-75-1 cells were transiently transfected with plasmids containing the TGF α 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 TGF α , MCF-7 or ZR-75-1 cells were infected with an amphotropic retrovirus containing the TGF α 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 TGF α 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 x 10⁴ 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 x 10⁶ 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.

TGF α 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 TGF α (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.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 04829-16 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) The Identification and Characterization of Human Genes Associated with Neoplasia		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Robert Callahan Giorgio Merlo Craig Cropp Timothy Mulligan	Chief, Oncogenetics Section Visiting Associate Senior Staff Fellow Medical Fellow	LTIB, DCBDC, NCI LTIB, DCBDC, NCI LTIB, DCBDC, NCI MB, DCT, 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 DCBD, NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 3.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The 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 compounded 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. In previous studies, we have detected frequent amplification of the <i>c-myc</i>, <i>int-2</i>, and <i>c-erbB2</i> proto-oncogenes and the frequent loss of heterozygosity (LOH) on chromosomes 1q, 3p, 11p and 13q. Our current results demonstrate that LOH also frequently occurs on chromosomes 1p, 17p, 17q, and 18q. LOH on chromosome 17q has a significant association ($p < 0.02$) with estrogen receptor negative tumors and LOH is associated ($p < 0.04$) with histopathological grade III tumors. We have also found associations between specific mutations. For instance, one subset of tumors could be defined by the frequent presence of LOH on chromosomes 11p, 17p, and 18q. Another subset of tumors contained LOH on chromosomes 1p, 13q, and 17q. These results suggest that different subsets of mutations, possibly acting in a complimentary way, are a consequence of the heterogeneous nature of the etiologic factors that provide the selective pressure for the clonal outgrowth of cells containing particular mutations during breast carcinogenesis. We have begun to identify the target genes affected by LOH. For example, on chromosome 17p, a likely candidate is the p53 gene. We have found that the remaining allele of p53, in tumors having LOH on 17p, may also contain a point mutation. On chromosome 17q, we have found LOH at the nm23 gene. This gene is tightly linked to the hereditary breast cancer locus.</p>		

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 located within the corresponding region of the homologous chromosome. In previous work we reported LOH on chromosomes 1q, 3p, 11p, and 13q. In our current work we have also found four additional regions of the genome which are affected by LOH in breast tumors. They are located on chromosomes 1p, 17p, 17q, and 18q and their frequency is 37%, 48%, 64%, and 34%, respectively. LOH on chromosome 17q has a significant association with estrogen receptor negative tumors ($p < 0.02$), where as, LOH on chromosome 18q is frequently ($p < 0.04$) found in histopathological grade III tumors. Although LOH on chromosomes 1p and 17p were not associated with any of the clinicopathological parameters available for this panel of tumors, in a separate study on a different panel of tumors LOH on chromosome 17p was found to be associated ($p < 0.01$) with breast tumors having a high proliferative index.

Since several of the mutations are associated with the more aggressive tumors the associations among the mutations has been considered. For the eleven mutations which we have found, there are 55 pairwise tests of independence. If all the mutations occurred independently of one another during tumor development, one would have expected 2.75 of them ($55 \times .05 = 2.75$) would frequently occur together at $P < 0.05$ in the same tumors. In fact, there are eleven pairs of mutations with P values < 0.05 that occur together in our tumor panel. In addition, particular subsets of mutations are suggested by these associations. For instance, LOH on chromosomes 11p, 17p, and 18q frequently occurs in the same tumor. Similarly, a different subset of tumors contain frequent LOH on chromosomes 1p, 17q, and 13q. Both subsets of tumors frequently contain LOH on chromosome 3p. These findings provide the basis for our working hypothesis that different subsets of mutations may make comparable contributions to the malignant phenotype. This concept of different subsets of mutations possibly acting in a complimentary way is consistent with the heterogeneous nature of the etiological factors that provide the selective pressure for mutations during breast carcinogenesis. Moreover, it suggests that it may be possible to determine how the different sets of mutations might collaborate in affecting cancer development.

At the present time the target genes for LOH are mostly unknown. The RB1 gene on chromosome 13q14 appears to be the target for LOH on this chromosome. Recently, we, in collaboration with Dr. John Minna, NCI, have found that tumors having LOH on chromosome 17p also contain point mutations in the p53 gene which is located in this region of the chromosome. Thus, in these tumors there is no expression of a normal p53 gene product. In other studies done in collaboration with Drs. Pat Steeg and Lance Liotta, NCI; we have found that one allele of the nm23 gene is lost in 64% of the breast tumors. This gene is located on chromosome 17q and is tightly linked to the locus for hereditary breast cancer. In experimental model systems loss of expression of this gene is associated with high metastatic potential for the tumor. Currently we are investigating whether the remaining allele of nm23 in tumors having LOH on chromosome 17q is altered by point mutations.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 CB 05148-12 LTIB
PERIOD COVERED		
October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)		
Mammary Tumorigenesis in Inbred and Feral Mice		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
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Daniel Gallahan	Senior Staff Fellow	LTIB, DCBDC, NCI
Antonio Marchetti	Visiting Fellow	LTIB, DCBDC, NCI
Fiamma Buttitti	Guest Worker	LTIB, DCBDC, NCI
COOPERATING UNITS (if any)		
Dr. Francesco Squartini, Univer. of Pisa, Pisa, Italy; Dr. Glenn Merlino, NCI; Dr. Gordon Peters, ICRF, London, England		
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Laboratory of Tumor Immunology and Biology		
SECTION		
Oncogenetics Section		
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with the mouse mammary tumor virus (MMTV) and have been bred for a high incidence of mammary tumors. MMTV appears to induce tumors by acting as an insertional mutagen that leads to the activation of a previously silent gene or the rearrangement of a normally expressed gene (<i>int</i> genes). We have found that the frequency with which different <i>int</i> genes are activated in mammary tumors depends on the particular strain of mice. For instance, 80% of the C3H mammary tumors contain a viral induced rearrangement of the <i>int-1</i> locus, whereas, in BALB/cfC3H mammary tumors the <i>int-1</i> gene is rearranged in only 30% of the tumors. This suggests that the inbreeding program has selected for the fixation of a host mutation which somehow compliments the action of <i>int-1</i> gene expression during tumor development. We have expanded our studies to include the feral CZECHII mouse strain, which lacks endogenous MMTV genomes in its germline but is congenitally infected with exogenous MMTV. Several viral induced preneoplastic hyperplastic outgrowth (HOG) lines have also been developed. Three of these have been found to have a viral insertion at <i>int-1</i>. A new <i>int</i> locus, designated <i>int-6</i>, has been found in a fourth HOG line. Many of the HOG lines spontaneously give rise to mammary tumors and, in two independent cases, the mice also contained metastatic lesions in their lungs. The primary tumors frequently contain additional viral insertions over those observed in the particular HOG line. Similarly, the metastatic tumors frequently contain additional viral insertions over those observed in the primary tumor and the particular HOG line. This suggests that we may be able to identify new <i>int</i> genes that are associated with the particular stages of malignant progression. We have shown in other studies that the <i>int-2</i> gene product can functionally replace bFGF and compete with it for cellular receptors.</p> <p>Work previously reported under Project number Z01CB 09027 has been incorporated into this project.</p>		

Major findings

Sixteen hyperplastic mammary outgrowth lines were established from Czech II mice infected with Czech II, GR or C3H mouse mammary tumor virus. Twelve of these lines have been stable for at least 4 transplant generations. Mammary tumors have developed stochastically within 9 of these lines, the other 3 have remained tumor-free up to this time. The cellular DNA from these lines and their tumors have been analyzed for MMTV DNA insertions which might result in the rearrangement of known *int* genes, e.g. *int-1*, *int-2*, and *int-3*. Three of the hyperplastic lines were found to have rearrangements at *int-1*. None of the other preneoplastic outgrowth lines had rearrangements in any of the known *int* genes. Therefore, these hyperplastic lines represent cellular populations which are potential candidates for revealing unrecognized *int* genes which are affected by MMTV insertional mutations. Comparison of the MMTV insertions in the tumors from the various lines with those present in the original hyperplastic outgrowth indicated that these tumor cell populations were clonal expansions of hyperplastic cells which had acquired new MMTV DNA insertions. In addition, metastatic lung nodules were observed in two mice bearing two separate preneoplastic lines, one with an *int-1* rearrangement and the other without rearrangements associated with known *int* genes. Individual metastatic nodules were transplanted to mammary fat pads and the resulting tumors were analyzed for MMTV DNA insertions. The results of these analyses indicated that each metastatic nodule was a clonal expansion of cells from the primary tumor which had occurred within the hyperplastic population. In addition, these tumor populations possessed the same MMTV integrations which had been acquired by those cells comprising the primary tumor. Preliminary studies indicate that some of these metastatic populations also contain additional MMTV insertional mutations. Some of these may be relevant to the progression to the more malignant metastatic phenotype. This observation, coupled with the fact that the number and position of MMTV insertions in the hyperplastic outgrowth lines were stable in succeeding generations, suggests that examining the flanking host sequences at these new insertions may reveal genes which are relevant to the progression of the preneoplastic phenotype to a malignant one. In fact, we have identified a new *int* locus for MMTV (designated *int-6*) in one of the preneoplastic hyperplastic outgrowth lines in which the other known *int* genes were unaffected. This locus is also rearranged in five independent MMTV induced mammary tumors.

The frequency with which the *int* genes are activated during mammary tumorigenesis is dependent in part on the host genetic background. For instance, in the high incidence C3H inbred mouse strain *int-1* is rearranged by MMTV in 80% of the tumors, whereas, in BALB/cf C3H mice this locus is altered by the virus in only 30% of the mammary tumors. Similarly, there is a significant difference in the frequency with which the *int-2* locus is rearranged by MMTV in RIII and BALB/cf RIII mammary tumors. This suggests that the selection for high tumor incidence during the inbreeding of these mouse strains has resulted the fixation of host mutations in their genetic backgrounds which compliment the action of the particular *int* gene during tumorigenesis.

We have begun to assess the biological activity of the different *int* gene products on mammary cells *in culture* and *in vivo*. The *int-2* gene is a member of the fibroblast growth factor (FGF) family of genes. We have been able to show that the *int-2* protein can functionally replace the requirement of bFGF by the human SW13 adrenal cortical

cells for growth in soft agar and can compete with bFGF for receptors on the cells. However, this required the addition of the mouse immunoglobulin signal peptide sequence to the *int-2* coding sequence for the protein to be secreted from the cells. Introduction of an activated *int-2* gene into the HC11 mouse mammary epithelial cell line also provide these cells soft agar growth capability. In these cells the artificial signal peptide was not required for secretion of the *int-2* protein.

In collaboration with Dr. Glenn Merlino, NCI, we have developed a transgenic mouse strain in which the transgene is a portion of the *int-3* gene linked to the MMTV LTR. Of the seven founders two were females; and one of these females developed a mammary tumor. Mammary tumors were also observed in two of the males. Expression of *int-3* also is associated with hyperplasia of the salivary glands and submucosal glands.

Publications

Valverius, EM, Ciardiello, F, Heldin, N-E, Blondel, B, Merlo, G, Smith, G, Stampfer, MR, Lippman, ME, Dickson, RB, and Salomon, DS. Stromal influences on transformation of human mammary epithelial cells overexpressing *c-myc* and SV40T, *J Cell Physiol* 1990; 145: 207-216.

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Marchetti, A, Robbins, J, Campbell, G, Buttitta, F, Squartini, F, Bistocchi, M, and Callahan, R. Host genetic background effect on the frequency of MMTV induced rearrangemnts of the *int-1* and *int-2* loci in mouse mammary tumors, *J Virol* 1991; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 09023-05 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
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, laboratory, and institute affiliation)		
Syed Kashmiri	Expert	LTIB, DCBDC, NCI
Benjamin Calvo	Staff Fellow	LTIB, DCBDC, NCI
Liming Shu	Visiting Associate	LTIB, DCBDC, NCI
Anna Maria Masci	Visiting Fellow	LTIB, DCBDC, NCI
William Shupert	BTP Fellow	LTIB, DCBDC, NCI
Patricia Horan Hand	Chemist	LTIB, DCBDC, NCI
Jeffrey Schlom	Chief	LTIB, DCBDC, NCI
COOPERATING UNITS (if any) M. Whitlow, Genex; J. Leddy Dow Chemical; E. Padlan, NIH, Laboratory of Molecular Biology		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Oncogenetics Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 6.8	PROFESSIONAL: 1.7	OTHER: 5.1
CHECK APPROPRIATE BOXES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The main objective of this research project is to genetically engineer immunoglobulin genes to generate useful reagents for <i>in vivo</i> localization and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) that selectively react with tumor-associated antigens. These include the carcinoembryonic antigen and a tumor-associated glycoprotein, TAG-72, which is present in a variety of carcinomas. The MAbs are currently being used in a number of diagnostic and therapeutic trials on breast, colon and ovarian cancers. In clinical tests, two such MAbs, B72.3 and CC49, which were raised against the tumor associated antigen TAG-72, have shown promise for being developed into diagnostic and therapeutic agents. The usefulness of mouse MAbs for <i>in vivo</i> therapy and diagnosis of human tumors, however, is limited because of their immunogenicity in patients. This problem can be reduced by replacing the constant region of the mouse antibody with the constant region of the human antibody, using recombinant DNA techniques. To that end, we have cloned the rearranged heavy and light chain variable regions as well as the cDNA copies of the heavy and light chain gene messages of the MAb, B72.3. Chimeric heavy and light chain immunoglobulin genes have been constructed, inserted separately into appropriate expression vectors, and stable chimeric antibody-producing cell lines were developed by introducing these constructs into both fibroblast and myeloma cell lines. Chimeric antibody produced either from cDNA clones or clones of the rearranged genes retained specificity and binding properties of the parental antibody.</p> <p>A novel type of recombinant molecule, a single chain antigen binding protein, has also been constructed and characterized. The molecule, also termed a single chain Fv, was constructed and expressed in <i>E. coli</i>, and is composed of a variable light chain amino acid sequence tethered to a variable heavy chain sequence by a designed peptide.</p>		

Major Findings

We have generated and characterized a recombinant/chimeric construct of murine $\gamma 1$ monoclonal antibody (MAb) B72.3, containing the murine variable region and a human $\gamma 1$ constant region [designated cB72.3($\gamma 1$)]. cB72.3 ($\gamma 1$) was generated by first isolating functionally rearranged V_H and V_L genes of B72.3 from partial genomic libraries in phage vectors. Construction of mouse-human chimeric heavy and light chain genes was performed by inserting restriction fragments carrying V_L and V_H regions of B72.3 into unique sites of expression vectors which contain sequences encoding constant regions of a human K and $\gamma 1$, respectively. The expression constructs were subsequently electroporated into SP2/0 ccells. The transfected SP2/0 murine cell line has been shown to synthesize cB72.3 ($\gamma 1$) at a level of 10-20 $\mu\text{g/ml}$. Reciprocal competition radioimmunoassays demonstrated that cB72.3($\gamma 1$), a previously described cB72.3($\gamma 4$), and native B72.3 (designated nB72.3) competed similarly. A rat anti-idiotypic MAb made against nB72.3 was shown to bind equally well to cB72.3($\gamma 1$) and to the nB72.3. Immunochemical studies of the nB72.3, cB72.3($\gamma 4$), and cB72.3($\gamma 1$) revealed slight differences in size among the three MAb forms on sodium dodecyl sulfate gels and revealed a higher isoelectric point for the cB72.3($\gamma 1$). Antibody-dependent cell-mediated cytotoxicity experiments using human lymphokine-activated killer effector cells indicated better tumor cell killing by the cB72.3($\gamma 1$) than the nB72.3 or cB72.3($\gamma 4$). Dual label studies of coinjected cB72.3($\gamma 1$) 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($\gamma 1$), cB72.3($\gamma 4$), 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($\gamma 1$) and the nB72.3 showed similar clearance curves, while the cB72.3($\gamma 4$) 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($\gamma 1$) may serve as a potentially useful diagnostic and/or therapeutic reagent.

We have conducted experiments which describe the first *in vivo* targeting of tumors with a single-chain antigen-binding protein. The molecule, which was constructed and expressed in *E. coli*, is a novel recombinant protein composed of a variable light-chain (V_L) amino acid sequence of an immunoglobulin tethered to a variable heavy-chain (V_H) sequence by a designed peptide. We show that this protein, derived from the DNA sequence of the variable regions of the antitumor monoclonal antibody B6.2, has the same *in vitro* antigen-binding properties as the B6.2 Fab' fragment. Comparative pharmacokinetic studies in athymic mice demonstrate much more rapid alpha and beta phases of plasma clearance for the single-chain antigen-binding protein than for the Fab' fragment, as well as an extremely rapid whole-body clearance. Half-life values for alpha and beta phases of single-chain antigen-binding protein clearance were 2.4 minutes and 2.8 hours, respectively, versus 14.8 minutes and 7.5 hours for Fab'. Furthermore, the single-chain antigen-binding protein molecule did not show accumulation in the kidney as did the Fab' molecule or as previously shown, the F(ab')₂ molecule. Despite its rapid clearance, the single-chain antigen-binding protein showed uptake in a human tumor xenograft comparable to that of the Fab' fragment, resulting in tumor to normal tissue ratios comparable to or greater than those obtained with the Fab' fragment. These studies thus demonstrate the *in vivo* stability of recombinant single-chain antigen-binding proteins and their potential in some diagnostic and therapeutic clinical applications in cancer and other diseases.

Publications

Hutzell, P, Kashmiri, S, Colcher, D, Primus, FJ, Horan Hand, P, Roselli, M, Finch, M, Yarranton, G, Bodmer, M, Whittle, N, King, D, Loullis, CC, McCoy, DW, Callahan, R, and Schlom, J. Generation and characterization of a recombinant/chimeric B72.3 (human $\gamma 1$), *Cancer Res* 1991;51:181-189.

Colcher, D, Bird, R, Roselli, M, Hardman, K, Johnson, S, Pope, S, Pantoliano, M, Milenic, D, and Schlom, J. *In vivo* tumor targeting of a recombinant single chain antigen binding protein, *J Natl Cancer Inst* 1990; 82:1191-1197.

Primus, FJ, Pendurthi, TK, Hutzell, P, Kashmiri, S, Slavin, D, Callahan, R, and Schlom, J. Chimeric B72.3 mouse-human ($\gamma 1$) antibody directs the lysis of tumor cells by lymphokine-activated killer cells, *Cancer Immunol Immunother* 1990;31:349-357.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 05216-20 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Site-Selective cAMP Analogs as Antineoplastics and Chemopreventivess		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Yoon S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBDC, NCI
Timothy Clair	Chemist	LTIB, DCBDC, NCI
Hiroshi Yokozaki	Visiting Fellow	LTIB, DCBDC, NCI
Alfredo Budillon	Visiting Fellow	LTIB, DCBDC, NCI
COOPERATING UNITS (if any) Dr. B. Jastorff, University Bremen, FRG; Dr. W.R. Miller, U. of Edinburgh, Scotland; Dr. S.O. Døskeland, U. of Bergen, Norway; Dr. G. Tortora, U. of Naples Medical School II, Naples, Italy.		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Cellular biochemistry section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) 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 <i>in vitro</i> and <i>in vivo</i> 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-C1-cAMP, the most potent site-selective cAMP analog, acts synergistically with granulocyte-macrophage colony-stimulating factor in the differentiation of HL-60 human promyelocytic leukemia cells. 8-C1-cAMP also holds clinical promise for immunodiagnosis and immunotherapy. 8-C1-cAMP acts alone and synergistically with interferon gamma (IFN- γ) to selectively increase expression of carcinoembryonic antigen in IFN- γ insensitive LS-174T human colorectal carcinoma cells <i>in vitro</i> and <i>in vivo</i> . 8-C1-cAMP 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-C1-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-C1-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. 8-C1-cAMP is now in preclinical phase I studies at NCI.		

Major Findings

In HL-60 leukemia cells the site-selective cAMP analog 8-C1-cAMP, at a dose of 5 μM produced growth inhibition with no signs of toxicity, whereas granulocyte-macrophage colony stimulating factor (GM-CSF) exerted an early transient increase of cell proliferation which was followed by differentiation towards monocytes. 8-C1-cAMP in combination with GM-CSF blocked the growth stimulation due to GM-CSF and demonstrated a synergistic effect on the differentiation of HL-60 cells. The early proliferative effect of GM-CSF was correlated with an increased expression of type I regulatory subunit of cAMP-dependent protein kinase ($\text{RI}\alpha$). Treatment with $\text{RI}\alpha$ antisense oligodeoxynucleotide suppressed the GM-CSF-inducible cell proliferation and differentiation. Conversely, an $\text{RII}\beta$ antisense oligodeoxynucleotide, which suppresses the $\text{RII}\beta$ and causes a compensatory increase in $\text{RI}\alpha$ level, greatly enhanced the early proliferative input and the differentiation induced by GM-CSF. These results provide an insight into the mechanism of action of GM-CSF and the rationale for a combination differentiation therapy with 8-C1-cAMP and GM-CSF.

Site-selective cAMP analogs inhibit the growth of multidrug resistant cell lines and suppress the expression of P-glycoprotein: Site-selective cAMP analogs induce growth inhibition and differentiation in a broad spectrum of human cancer cell lines. Such effects of analogs correlate with the analog's ability to suppress type I cAMP-dependent protein kinase (PKA) and enhance type II PKA expression. PKA has been implicated in some multidrug resistance (mdr) phenotypes. We have examined the effect of 8-C1-cAMP and N^6 -benzyl-cAMP, the two most potent cAMP analogs, on the growth of mdr cell lines and the expression of p-glycoprotein (pgp), the plasma membrane drug efflux pump. The mdr cell lines tested include those containing pgp, HL-60/VCR, KB-V1 and 3T3/MDR, and one devoid of pgp, HL-60/ADR. 8-C1-cAMP exerted potent growth inhibition of both pgp-associated and -unassociated mdr cells with an IC_{50} of 0.1 - 10 μM at 3-4 days with no sign of cytotoxicity. Growth inhibition accompanied a reduction in pgp expression. N^6 -benzyl-cAMP exhibiting an IC_{50} of 2-30 μM at 3-4 days showed a greater potency of growth inhibition toward pgp-unassociated mdr cells than pgp-associated mdr cells or the parent non-mdr cells. These results show that site-selective cAMP analogs are potent growth inhibitors of mdr cells and suggest that different mechanisms of action of cAMP account for the growth inhibition of pgp-associated vs -unassociated mdr cells.

In some mdr cell lines, cAMP-dependent protein kinase (PKA) has been implicated in the mdr phenotype. To ascertain the role of PKA in mdr, the sensitivity of HL-60, HL-60/ADR and HL-60/VCR cells to ADR was determined in the presence and absence of 8-C1-cAMP, a site B-selective analog which shows specificity for binding to type II PKA via the regulatory subunit, RII. The IC_{50} of 8-C1-cAMP in HL-60, HL-60/ADR and HL-60/VCR cells after 48 h was 10, 17 and 18 μM , respectively. The IC_{50} of ADR in HL-60, HL-60/ADR and HL-60/VCR cells after 48 h was 0.03, 12 and 1 μM , respectively. At non-inhibitory concentrations (IC_{10}) of 8-C1-cAMP, the IC_{50} of ADR after 48 hr was potentiated 1.2-, 13.8- and 2.3-fold in HL-60, HL-60/ADR and HL-60/VCR cells, respectively. The marked synergism between 8-C1-cAMP and ADR suggests that type II PKA plays an important role in mdr cells, particularly in ADR-resistant HL-60 cells which lack pgp. The effect of 8-C1-cAMP on the levels of type I and II PKA and RI and RII, as well as on factors regulating cAMP-responsive promoter elements is being assessed.

Treatment of human colorectal tumor cells (LS-174T, HT-29, and WiDr) with analogs of cyclic AMP (cAMP) (dibutyryl-cAMP and 8-C1-cAMP) selectively enhances the expression of carcinoembryonic antigen (CEA). Dose and temporal kinetics results revealed that 8-C1-cAMP was approximately 100-fold more potent than dibutyryl-cAMP for increasing CEA expression. Results demonstrated that 8-C1-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-C1-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-C1-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-C1-cAMP treatment, suggesting some selectivity for CEA regulation by this cAMP analog. *In vivo* administration of 8-C1-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-C1-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-C1-cAMP treatment regulates CEA expression through another cellular pathway which may involve cAMP-dependent protein kinase.

Publications

- Cho Chung, YS. Perspectives in cancer research. Role of cAMP receptor proteins in growth, differentiation and suppression of malignancy: New approaches to therapy, *Cancer Res* 1990; 50:7093-7100.
- 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* (in press).
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- Cho Chung, YS, Tortora, G, Pepe, S, Yokozaki, H. IVth Conference on Differentiation therapy: The differentiation of HL-60 human promyelocytic leukemia by 8-C1-cAMP and rhGM-CSF in combination, Rome, Italy: Raven Press (In press.)
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 08281-09 LTIB
PERIOD COVERED October 1, 1990 to September 30, 1991		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanism of cAMP in Growth Control and Differentiation: Gene Regulation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
Yoon-S. Cho-Chung	Chief, Cellular Biochemistry Section	LTIB, DCBDC, NCI
Hiroshi Yokozaki	Visiting Fellow	LTIB, DCBDC, NCI
Timothy Clair	Chemist	LTIB, DCBDC, NCI
Christian Rohlf	Special Volunteer	LTIB, DCBDC, NCI
COOPERATING UNITS (if any) Dr. M. Medniekes, U. of Chicago, Wyler Children's Hospital, Chicago, IL; Dr. Tore Jahnsen, Institute of Pathology, Rikshospitalet, Oslo, Norway; Dr. R. Tahara, Hiroshima Univ. Med. School, Hiroshima, Japan; Dr. D. Ogreid, U. of Bergen, Norway.		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Cellular Biochemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS 2.5	PROFESSIONAL 2.0	OTHER 0.5
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unrepduced 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 incontrolled outgrowth: cancer.		
<p>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 the RII gene by a retroviral vector.</p> <p>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 all growth and differentiation and provide new approaches to the treatment of cancer.</p>		

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-[³²P]cAMP showed that peak 1 contained RI α (48 KDa), while peak 2 contained RII β (50KDa). 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 (~40KDa) in peak 1. These results were confirmed when cells were treated with 8-Cl-[³H]-cAMP. Thus, a truncated RI α subunit retaining the binding ability to both C subunit and cAMP is involved in the suppression of PKA-I expression by 8-Cl-cAMP.

An antisense oligodeoxynucleotide targeted against RII β subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemic cells without affecting phorbol ester effects: The RII β regulatory subunit of cAMP-dependent protein kinase has been hypothesized to play an important role in the growth inhibition and differentiation induced by site-selective cAMP analogs in human cancer cells, but direct proof of this function has been lacking. To address this issue, HL-60 human promyelocytic leukemic cells were exposed to RII β antisense synthetic oligodeoxynucleotide, and the effects on cAMP-induced growth regulation were examined. Exposure of these cells to RII β antisense oligodeoxynucleotide resulted in a decrease in cAMP analog-induced growth inhibition and differentiation without apparent effect on differentiation induced by phorbol esters. The loss in cAMP growth regulatory function correlated with a decrease in both basal and induced levels of RII β protein. Exposure to RII β sense, RI α and RII α antisense, or irrelevant oligodeoxynucleotides had no such effect. These results show that the RII β regulatory subunit of protein kinase plays a critical role in the cAMP-induced growth regulation of HL-60 leukemic cells.

Differentiation of HL-60 leukemia by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase: A marked decrease in the type I cAMP-dependent protein kinase regulatory subunit (RI α) and an increase in the type II protein kinase regulatory subunit (RII β) correlate with growth inhibition and differentiation induced in a variety of types of human cancer cells, *in vitro* and *in vivo*, by site-selective cAMP analogs. To directly determined whether RI α is a growth-inducing protein essential for neoplastic cell growth, human HL-60 promyelocytic leukemia cells were exposed to 21-mer RI α antisense oligodeoxynucleotide and the effects on cell replication and differentiation were examined. The RI α antisense oligomer brought about growth inhibition and monocytic differentiation, by-passing the effects of an exogenous cAMP analog. These effects of RI α antisense oligodeoxynucleotide correlated with a decrease in RI α receptor and an increase in RII β

receptor level. The growth inhibition and differentiation were abolished however, when these cells were exposed simultaneously to both RI α and RII β antisense oligodeoxynucleotides. The RII β antisense oligodeoxynucleotide alone specifically blocks the differentiation inducible by cAMP analogs. These results provide direct evidence that RI α cAMP receptor plays a critical role in neoplastic cell growth and that cAMP receptor isoforms display specific roles in cAMP-regulation of cell growth and differentiation.

Type II regulatory subunit of protein kinase restores cAMP-dependent transcription in a cAMP-unresponsive cell line: cAMP-dependent protein kinase appears to play a role in cAMP-induced gene expression in mammalian cells. There exist two major types of cAMP-dependent protein kinase, type I and type II, which are distinguished by their regulatory subunits, RI and RII respectively. We investigated the role of type I and type II protein kinase in the cAMP-induced gene expression by either stable- or co-transfection of RI α , RII α , or RII β gene in an expression vector together with somatostatin-chloramphenicol acetyl-transferase (SS-CAT) fusion gene using a cAMP-unresponsive mutant pheochromocytoma cell line (A126-1B2). Introduction of the RII β gene restored the capability of these cells to induce the SS-CAT gene expression in response to forskolin stimulus, and induced a changed morphology which resembled that of wild-type. The RII α gene also induced SS-CAT gene expression but to a lesser degree than that achieved by the RII β gene, whereas the RI α gene had no effect. The induction of SS-CAT gene expression by the RII β gene was specifically blocked by the 21-mer RII β antisense oligodeoxynucleotide. These results show for the first time that type II but not type I regulatory subunit of cAMP-dependent protein kinase is essential for a cAMP-induced gene transcription.

Publications

- Tortora, G, Cho-Chung, YS. Type II regulatory subunit of protein kinase restores cAMP-dependent transcription in a cAMP-unresponsive cell line, *J Biol Chem* 1990;265:18067-18070.
- Cho-Chung, YS. Perspectives in cancer research. Role of cAMP receptor proteins in growth, differentiation and suppression of malignancy: New approaches to therapy, *Cancer Res* 1990;50:7093-7100.
- Tortora, G, Yokozaki, H, Pepe, S, Cho-Chung, YS. Differentiation of HL-60 leukemia by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase, *Proc Natl Acad Sci USA* 1991;88:2011-2015.
- Cho-Chung, YS, Clair, T, Tortora, G, Yokozaki, H, Pepe, S, Minireview: Suppression of malignancy targeting the intracellular signal transducing proteins of cAMP: The use of site-selective cAMP analogs, antisense strategy and gene transfer, *Life Sci* 1991;48:1123-1132.
- Cho-Chung, YS, Clair, T, Tortora, G, Yokozaki, H. Review: Role of site-selective cAMP analogs in the control and reversal of malignancy, *Pharmacology and Therapeutics* (in press).

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

PROJECT NUMBER

Z01 CB 08907-08 OD

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Regulation of Immune Response to Tumor Cells

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

PI:	C.C. Ting	Senior Investigator	OD DCBDC NCI
Others:	M.E. Hargrove	Microbiologist	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:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

1. Glutathione (GSH) regulation of the CD3-AK response. GSH, a tripeptide, up-regulates the differentiation of preactivated IL-2 dependent activated killer cells, namely LAK and CD3-AK. GSH acts through the up-regulation of the utilization of IL-2. In contrast, GSH down-regulates the generation of LAK and CD3-AK in resting T cells. Flow cytometry analysis reveals that GSH down-regulates the expression of IL-2 receptors and T cell receptor CD3 on the surface of activated killer cells. It appears that through this effect the activation of T cells in resting lymphocytes was suppressed. Therefore, GSH exerts a dichotomous effect on the regulation of the generation of activated killer cells.

2. Cytokine regulation of the generation of activated killer cells. The generation of CD3-AK is largely PKC (protein kinase C) dependent. After depleting PKC by prolonged treatment with PMA, splenocytes lost their ability to generate CD3-AK cells in culture medium which was supplemented with IL-2. However, adding IL-4 in the cultures restored the responsiveness to α CD3 activation. Therefore, IL-4 appears to play a major role in a PKC-independent pathway for the generation of CD3-AK cells.

Major Findings:

I. Regulation by glutathione (GSH) of the differentiation of primary activated lymphocytes.

A) Influence of GSH on the preactivated primary killer cells. The activated killer cells CD3-AK were generated by activation of normal mouse splenocytes with anti-T cell receptor-CD3 antibody. The cytolytic activity of CD3-AK was IL-2 dependent and was decreased by α IL-2 or α IL-2/ α IL-4 antibodies. BSO (L-buthionine-sulfoximine), an inhibitor of de novo GSH synthesis, decreased GSH levels and inhibited the cytolytic activity of CD3-AK. It is known that GSH regulates IL-2 activity, and through this regulatory effect GSH may affect the differentiation of primary cytotoxic lymphocytes. These results could be reproduced in LAK cells, another line of IL-2 dependent killer cells.

B) Influence of GSH on the activation of killer cells in resting lymphocytes. In contrast to the up-regulation by GSH of the preactivated killer cells, GSH down-regulates the generation of CD3-AK and LAK cells from resting lymphocytes. This is attributed to the down-regulation of TCR and IL-2R of these killer cells or their precursors. Thus, GSH exerts a dichotomous effect on the regulation of IL-2 dependent killer cells, depending on the state of activation.

II. Cytokines as the co-stimulatory signal for activation of killer cells in effector phase.

After initial activation, further culturing CD3-AK cells in IL-2 alone resulted in the generation of killer cells (CD3-AK) to mediated slow lysis. In effector phase for CD3-AK cells to mediated slow lysis, a PKC-dependent activation phase preceded the lytic phase. IL-2 and IL-4 are involved in the early activation phase, and TNF is involved in the late activation phase to activate CD3-AK. When this process is completed, the lytic machinery is turned on to initiate the lytic reaction and completes the lytic process.

III. IL-4 regulation of CD3-AK response. The major pathway for generating CD3-AK cells is PKC-dependent. However, a PKC-independent pathway does exist and accounted for one third of total CD3-AK response. This pathway is primarily regulated by IL-4. In this pathway, IL-4 is the key element to switch on the CD3-AK response, probably through switching on the genes that are coded for the production of cytolytic granules perforin/cytolysin which are responsible for mediating the lytic reactions.Proposed Course of Research:

1. To determine the role of cytokines in the regulation of cytotoxic response and proliferative response to the stimulation of T cell receptor-CD3 complex.

2. To determine the role of biological response modifiers (e.g., PMA) and cytokines on the regulation of gene expression in response to activation of TCR-CD3.
3. To continue the study on the mechanism of lymphocyte-mediated cytolysis by employing our fast lysis/slow lysis model.
4. To determine the role of IL-4 and CD3-AK combined therapy in treating experimental tumors.

Publications:

Liang SM, Liang CM, Hargrove ME, Ting CC. Regulation by glutathione of the effect of lymphokines on differentiation of primary activated lymphocytes. Influence of glutathione on cytotoxic activity of CD3-AK. J Immunol 1991;146:1909.

Ting CC, 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; in press.

CONTRACT RESEARCH SUMMARY

Title: Provide Computer Programming Support Services for the Experimental Immunology Branch

Principal Investigator: Lorenzo F. Expositor
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: Installation, configuration, and testing of server devices/software to allow each of three EIB VAX-series computers direct access to multiple hardcopy units has been completed. Work necessary to install, configure, and test 6 BETA-Test versions of Becton-Dickinson (BDIS) data acquisition software and 3 versions of BETA-Test BDIS protocol entry software has been performed. Software has been written, installed, and tested which provides automated; a) user account creation; b) data-directory structure creation; c) network file transfer during data collection; and d) hardcopy printout of data file information during data collection. The Laboratory Analysis Package (LAP) for analysis of histogram data files was installed, configured and tested. LAP code has been modified and multiple command files have been written to modify LAP for use in the flow cytometry laboratory.

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 91 Funds: \$69,695. D

CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Norman Beaudry
Performing Organization: Hazleton Biotechnologies Corp.
Vienna, Virginia

Contract Number: NO1-CB-7-1010
Starting Date: 6/30/87 Expiration Date: 6/29/93

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-2R α 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: 1991 Funds: \$266,357

B

CONTRACT RESEARCH SUMMARY

Title: Transplantation, Induction, and Preservation of Plasma Cell Tumors
in Mice and the Maintenance of Special Strains

Principle Investigator: Judith Wax
Performing Organization: Hazleton Laboratories
City and State: Rockville, MD

Contract Number: N01-CB-71085
Starting Date: 02-01-87 Expiration Date 01-31-92

Goal: Induction, transplantation, preservation and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of (congenic) strains of mice to find genes controlling susceptibility and resistance to the induction of plasma cell tumors by pristane; maintenance of wild mouse colony.

Approach: Maintain a closed conventional colony of inbred and congenic strains of mice, 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 has acquired the capability to test in mouse for RFLP polymorphisms and thereby advance the congenics and carry out quality control studies on the congenics. 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 91 Funds: \$715,927

B

CONTRACT RESEARCH SUMMARY

Title: Maintenance and Development of Inbred and Congenic Resistant Mouse Strains

Principal Investigator: Mr. David Bernard
Performing Organization: Hazleton Washington, Inc.
City and State: Rockville, MD

Contract Number: N01-CB-71091
Starting Date: 2/1/87
Expiration Date: 9/30/91

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintained a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing was carried out at each generation by cytotoxicity typing of breeders from each strain. Alloantisera were raised between mouse strains to assist in this quality control typing, and sera and animals were shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing were all highly satisfactory. The back-crossing program which was previously used for all congenic resistant strains in order to keep the backgrounds of these strains identical was discontinued. This program had been very satisfactory and had led to sixteen new recombinant H-2 haplotypes. However, financial considerations made it necessary to stop this procedure.

Fusions for hybridoma production were performed by the contractor, with the purpose of developing monoclonal antibodies to histocompatibility antigens. Antisera for histocompatibility antigen typing were prepared in various combinations and were found to be excellent reagents. A series of strain-restricted typing sera were produced in order to distinguish each strain in the colony from all other strains. Shipping of animals and sera to collaborating investigators at NIH and elsewhere was satisfactory. The animals shipped were generally of excellent health and provided breeding stock for the production of experimental animals in numerous laboratories. Computerization of records and reports has been performed and has led to efficient data handling. Hybridoma reagents were produced, stored and shipped starting with cell lines developed by the Project Officer.

Significance to Cancer Research: This animal facility was needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals made possible research on individual histocompatibility antigens and, in particular, the role of the major histocompatibility complex in the transplantation of tissues and cells and in the immune response to cancer.

Project Officers: Dr. D. H. Sachs, Mr. J. S. Arn, Dr. J. R. Wunderlich
Program: Immunology Resource
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 91 Funds: \$255,691

B

CONTRACT RESEARCH SUMMARY

Title: Maintenance of an Animal Holding Facility and Provision of Attendant Research Services

Principal Investigator: Ms. Leanne DeNenno

Performing Organization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: N01-CB-85607

Starting Date: 11/01/87 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, 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 and rats 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 have been established and 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 91 Funds: \$792,593 B

CONTRACT RESEARCH SUMMARY

Title: Facility for Preparing and Housing Virus Infected Mice, Genetically Manipulated Mice, and Chimeric Mice

Principal Investigator: Mr. Kinta Diven
Performing Organization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: N01-CB-8-5608
Starting Date: 09/30/88 Expiration Date: 09/29/93

Goal: Perform a variety of in vivo experiments in mice (up to a colony of 3600 animals) that cannot be performed on NIH campus as designated by the Project Officer. These experiments are to be performed in support of intramural research programs in the Experimental Immunology Branch, NCI.

Approach: Experiments are to be performed involving the transfer of normal and neoplastic cells, infection with virus, inoculations of combinations of cells and virus, irradiation with a-rays, preparation of radiation chimeric mice, thymus transplants and the breeding, care, and manipulation of SCID mice. Protocols and details of experiments are to be carried as directed by the Project Officer.

Progress: Experiments have been performed that involve: infection of mice with cytomegalovirus; 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'91: Mice received, 6000; mice born and weaned at contract site, 820; radiation chimeras prepared, 810; bone marrow prepared from 600 mice, spleen cell suspensions prepared from 300 mice; i. p. inoculations, 1400; s.q. inoculations, 70; foot pad injections, 40; intranasal injections, 70; tail bleeds, 1000; palpations, 300; ELISA tests, 100 samples; ascities harvested from 270 mice; viral plaque assays, 15; cytomegalovirus prepared, 350 ml; influenza A virus prepared, 200 ampules.

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 infections agents in NIH animal colonies. All of the protocols used in the facility related to viral infection, genetic manipulation of hemotopoietic cells, immunological resistance, models of immune deficiency, and reconstitution of the immune system.

Project Officer: Dr. Gene Shearer
Program: Immunology Resource
Technical Review Groups: Intramural Support Contract Subcommittee A
FY 91 Funds: \$474,504 EST. B

CONTRACT RESEARCH SUMMARY

Title: Feral Mouse Breeding Colony

Principal Investigator:
Performing Organization:
City and State:

Ms. Evelyn Hogg
Hazelton Laboratories America, Inc.
Rockville, MD

Contract Number: N01-CB-95621
Starting Date: 12/01/88

Expiration Date: 11/30/91

Goal: Induction of mammary tumors with biological (hormones, 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. Two transgenic mouse lines are also being maintained. Both strains have the FVB inbred mouse genetic background, one strain contains the alpha-TGF transgene and the other contains the MMTV LTR-int-3 transgene. 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. The int-3 transgenic mouse line has been successfully bred and maintained, females are incapable of lactation and the males are sterile. Analysis of the females of this transgenic strain have provide several insights into mammary gland development and the potential role of int-3 in mammary tumorigenesis. A new int gene (designated int-6) has been identified in a CzechII mammary hyperplastic outgrowth line. The expression of this gene is activated by MMTV insertional mutagenesis. Activation of this gene has been detected in five independent CzechII mammary tumors. Single cell suspensions of primary mammary epithelial cells infected with retroviral shuttle vectors containing the int-1 and int-2 genes have been introduced into cleared mammary fat-pads to determine their effect on mammary gland development and tumorigenesis.

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 91 Funds: \$151,446

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